Exhibit #3

Request for Inter Partes Reexamination
of U.S. Patent No. 8,067,381

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Patent Owner:	Natural Alternatives International, Inc.
Patent No.:	8,067,381
Issued:	November 29, 2011
For:	*METHODS AND COMPOSITIONS FOR INCREASING THE ANAEROBIC WORKING CAPACITY IN TISSUES*

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REQUEST FOR INTER PARTES REEXAMINATION UNDER 37 C.F.R. §1.915

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EXHIBITS

Exhibit A	Timeline showing the pendency periods of prior applications.
Exhibit B	[First] Preliminary Amendment dated March 22, 2004, U.S. Patent Appln. Ser. No. 10/717,217 ("Fifth Application")
Exhibit C	[Second] Preliminary Amendment dated September 2, 2008, U.S. Patent Appln. Ser. No. 10/717,217 ("Fifth Application")
Exhibit D	Request For Corrected Updated Filing Receipt dated October 1, 2008, U.S. Patent Appln. Ser. No. 10/717,217 ("Fifth Application")
Exhibit E	Corrected Filing Receipt dated February 5, 2009, U.S. Patent Appln. Ser. No. 10/717,217 ("Fifth Application")
Exhibit F	U.S. Patent No. 7,504,376 ("Fifth Patent")
Exhibit G	U.S. Patent No. 7,825,084 ("Sixth Patent")
Exhibit H	First Preliminary Amendment dated August 22, 2011, U.S. Patent Appln. Ser. No. 13,215,073 ("Seventh Application")
Exhibit I	Markman Order dated May 31, 2011 issued by Chief Judge Sleet, U.S. District Court for the District of Delaware, Construing the terms of U.S. Patent No. 5,965,596 ("First Patent"), U.S. Patent No. 6,172,098 ("Second Patent") and U.S. Patent No. 6,426,361 ("Third Patent")
Exhibit J	Asatoor, et al., Intestinal absorption of carnosine and its constituent amino acids in man, Gut, 1970, Vol. II, pp. 250-254 ("Asatoor")
Exhibit K	EPO Publication No. 0280593B1, June 12, 1991, ("EP '593")
Exhibit L	Gardner, et al., Intestinal Absorption of the Intact Peptide Carnosine in Man, and Comparison with Intestinal Permeability to Lactulose, Journal of Physiology (1991), Vol. 439, pp. 411-422 ("Gardner")
Exhibit M	U.S. Patent No. 5,869,068, De Lacharriere et al. ("De Lacharriere '068") and U.S. Patent No. 5,976,559, De Lacharriere et al. ("De Lacharriere '559")
Exhibit N	Wu, et al., Proximate Composition, Free Amino Acids and Peptides Contents in Commercial Chicken and Other Meat Essences, Journal of Food and Drug Analysis, (2002) Vol. 10 No. 3, 2002, pp. 170-177 ("Wu")
Exhibit O	Li, et al., Bioactivities of Chicken Essence, Journal of Food Science, 2012, 77: R105-R110 ("Li")
Exhibit P	Final Office Action dated August 15, 2001, U.S. Patent Appln. Ser. No. 09/757,782 ("Third Application").
Exhibit Q	EPO Publication No. 0449787A2, March 19, 1991, Setra S.r.l ("EP'787)
Exhibit R	Bauer, et al., Biosynthesis of carnosine and related peptides by skeletal muscle cells in primary culture, Eur. J. Biochem. Vol. 219, pp. 43-47. (1994) ("Bauer")
Exhibit S	Bakardjiev, et al., Transport of ß-Alanine and biosynthesis of carnosine by skeletal muscle cells in primary culture, Eur. J. Biochem. 225, 617-623 (1994) ("Bakardjiev")
Exhibit T	Casey, et al., Creatine ingestion favorably affects performance and muscle metabolism during maximal exercise in humans, Am. J. Physiol. 271 (Endocrinol. Metab. 34): E31-E37, 1996. ("Casey")

Exhibit U	Reaction rate tends to increase with concentration – phenomenon explained by collision theory, Chemical kinetics, Wikipedia.org ("Wikipedia")
Exhibit V	Biolo, et al., Insulin action on protein metabolism, Baillière's Clinical Endocrinology and Metabolism, Vol. 7, No. 4, October 1993 ("Biolo")

CLAIMS FOR WHICH REEXAMINATION IS REQUESTED AND FOR WHICH THERE IS A REASONABLE LIKELIHOOD <u>THAT REQUESTOR WILL PREVAIL</u>

1. Claims 1-14 Are Anticipated By U.S. Patent No. 5,965,596
2. Claim 1 is Anticipated by Asatoor
3. Claim 1 is Anticipated by EP '593
4. Claim 1 is Anticipated by Gardner
5. Claim 1 is Anticipated by De Lacharriere U.S. Patents Nos. 5,869,068 and 5,976,559
6. Claim 1 is Anticipated by Wu
7. Claim 1 is Anticipated by Li
8. Claim 1 is Obvious Over Setra in view of Asatoor
9. Claim 1 is Obvious Over Setra in view of Gardner
10. Claim 1 is Obvious Over Setra in view of Gardner and Asatoor
11. Claim 1 is Obvious Over Setra in view of Bauer
12. Claim 1 is Obvious Over Setra in view of Bakardjiev
13. Claim 1 is Obvious Over Setra in view of The State of the Art Including Wikipedia
14. Claims 2, 3, 4, 5, 7, 8, 10, 11, 12, 13 and 14 Are Obvious Over Setra In View of Asatoor
15. Claims 2, 3, 4, 5, 7, 8, 10, 11, 12, 13 and 14 Are Obvious Over Setra In View of Gardner
16. Claims 2, 3, 4, 5, 7, 8, 10, 11, 12, 13 and 14 Are Obvious Over Setra In View of Gardner and Asatoor
17. Claims 2, 3, 4, 5, 7, 8, 10, 11, 12, 13 and 14 Are Obvious Over Setra In View of Bauer
18. Claims 2, 3, 4, 5, 7, 8, 10, 11, 12, 13 and 14 Are Obvious Over Setra In View of Bakardjiev
19. Claims 2, 3, 4, 5, 7, 8, 10, 11, 12, 13 and 14 Are Obvious Over Setra In View of The State of the Art Including Wikipedia
20. Claim 6 is Obvious over Setra In View of Asatoor and Further In View of Biolo
21. Claim 6 is Obvious over Setra In View of Gardner and Further In View of Biolo
22. Claim 6 is Obvious over Setra In View of Gardner and Asatoor and Further in View of Biolo
23. Claim 6 is Obvious over Setra In View of Bauer and Further In View of Biolo
24. Claim 6 is Obvious over Setra In View of Bakardjiev and Further In View of Biolo
25. Claim 6 is Obvious over Setra In View of The State of the Art Including Wikipedia and Further In View of Biolo
26. Claim 9 is Obvious over Setra in View of Asatoor and Further in View of Casey
27. Claim 9 is Obvious over Setra in View of Gardner and Further in View of Casey
28. Claim 9 is Obvious over Setra in View of Gardner and Asatoor and Further In View of Casey
29. Claim 9 is Obvious over Setra in View of Bauer and Further in View of Casey
30. Claim 9 is Obvious over Setra in View of Bakardjiev and Further In View of Casey
31. Claim 9 is Obvious over Setra in View of The State of the Art Including Wikipedia and Further In View of Casey.

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Patent Owner:	Natural Alternatives International, Inc.
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REQUEST FOR INTER PARTES REEXAMINATION UNDER 37 C.F.R. §1.915

I. Introduction

This request for reexamination of Patent No. 8,067,381 (the "'381 Patent"), by Woodbolt Distribution, LLC ("Requestor"), is based upon several prior art references. One of these references by itself anticipates each and every claim of the '381 Patent. Other references by themselves anticipate claim 1 of the '381 Patent and in combination with additional references render all of the claims obvious. As will be demonstrated in this Request, there is a reasonable likelihood that Requester will prevail with respect to all of the claims of the '381 Patent, in view of the references cited and discussed.

In Section II below, Requestor demonstrates that the asserted claim to priority of the '381 Patent is defective and that the effective filing date of the '381 Patent, under 35 U.S.C. §120, is no earlier than April 10, 2003, not August 12, 1997 as indicated on the face of the '381 Patent.

The '381 Patent is the Seventh Patent in the series of applications and patents listed in the "Related U.S. Application Data" set forth on page 1. The claim to priority is defective because in the Fifth Application of the series, applicants deliberately and expressly terminated their claim

to the priority of the first four applications.[1] Despite that disclaimer, in the Sixth Application and in the Seventh Application which issued as the '381 Patent, applicants improperly asserted §120 priority claims to the First, Second, Third and Fourth applications.

Having broken the chain of priority between the Fourth and Fifth Applications, applicants' effective filing date under §120, for the application which issued as the '381 Patent, is no earlier than the April 10, 2003 filing date of the Provisional Application for the Fifth Application, not the First Application. A chart depicting these events is on page 6.[2]

The priority claims in the Sixth and Seventh Applications were defective for another reason. Each of these claims to priority recited that the Sixth Application was a continuation-in-part of the Fourth Application, even though the Fourth Application issued as a patent more than four years before the Sixth Application was filed, and therefore was not "co-pending" with it.

[1] The respective Applications and Patents in sequence, as referred to herein (e.g. First, Second, ...Seventh), with their correct earliest §120 priority dates, are as follows:

Appln/Pat.	Ser. No.	Filing Date	Issue Date	Patent No.	Correct Earliest §120 Date
First	08/909,513	Aug. 12, 1997	10/12/1999	5,965,596	August 12, 1997
Second	09/318,530	May 25, 1999	1/9/2001	6,172,098	August 12, 1997
Third	09/757,782	Jan. 9, 2001	7/30/2002	6,426,361	August 12, 1997
Fourth	10/209,169	Jul. 30, 2002	1/20/2004	6,680,294	August 12, 1997
Provisional	60/462,238	April 10, 2003			
Fifth	10/717,217	Nov. 18, 2003	3/17/2009	7,504,376	April 10, 2003
Sixth	12/231,240	Aug. 22, 2011	11/2/2010	7,825,084	April 10, 2003
Intermediate	12/806,356	Aug. 30, 2010			April 10, 2003
Seventh	13/215,073	Aug. 22, 2011	11/29/2011	8,067,381	April 10, 2003

[2] Also attached as Exhibit A is a timeline showing the pendency periods of these applications.

In Section III, Requestor demonstrates that the '381 Patent is invalid because the subject matter claimed in the '381 Patent is anticipated under 35 U.S.C. §102(b) by applicants' First Patent and Second Patent, both of which issued on earlier applications within the family of applications.

In Section IV, Requestor demonstrates that the composition of claim 1 of the '381 Patent is anticipated under 35 U.S.C. § 102(b) by each of several references not of record in the examination of the '381 Patent. Requestor also demonstrates that the composition of claim 1 is anticipated under 35 U.S.C. §§ 102(b) or 102(e) by De Lacharriere, U.S. Patent No. 5,976,559. That reference was relied upon by the Examiner in the prosecution of the Third Patent to finally reject claims virtually identical in substance to claim 1 of the '381 Patent. Although made of record by applicants in the prosecution of the '381 Patent, it was not applied by the Examiner. Finally, Requestor demonstrates that the compositions of claims 1-14 are obvious over multiple references, most of which were not of record during the prosecution of the '381 Patent.

II. The Earliest Date to Which Patent No. 8,067,381 Can Claim Priority is April 10, 2003

A. Applicants Broke the Chain of Priority Between the Fourth and Fifth Applications

Page 1 of the '381 Patent, in the section "Related U.S. Application Data," purports to claim §120 priority to the First through Sixth and Intermediate Applications, and to the Provisional Application. For convenience, that purported §120 priority claim is set forth as follows:

> Continuation of application No. 12/806,356, filed on Aug. 10, 2010 [Intermediate Application],
>
> which is a continuation of application No. 12/231,240, filed Aug. 29, 2008, now Pat. No. 7,825,084 [Sixth Application],

> which is a continuation of application No. 10/717,217, filed on Nov. 18, 2003, now Pat. No. 7,504,376, [Fifth Application] and a continuation-in-part of application No. 10/209,169, filed on Jul. 30, 2002, now Pat. No. 6,680,294 [Fourth Application],
>
> which is a continuation of application No. 09/757,782, filed on Jan. 9, 2001, now Pat. No. 6,426,361 [Third Application],
>
> which is a continuation of application No. 09/318,530, filed on May 25, 1999, now Pat. No. 6,172,098 [Second Application],
>
> which is a division of application No. 08/909,513, filed on August 12, 1997, now Pat. No. 5,965,596 [First Application].
>
> Provisional application No. 60/462,238, filed on Apr. 10, 2003 [Provisional Application].[3]

The Seventh Patent ('381 Patent) purports to claim priority under §120 to the First through Fourth Applications through the Fifth Application.[4] This asserted priority is incorrect because in the Fifth Application applicants deliberately and expressly deleted their claim to the First through Fourth Applications, claiming only the benefit of the Provisional Application filed April 10, 2003.

A [First] Preliminary Amendment dated March 22, 2004 in the Fifth Application originally asserted a priority claim through the Fourth, Third and Second Applications back to the First Application.[5] But, in a [Second] Preliminary Amendment and Request for Continued

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[3] The identification of each Application has been added in brackets "[ ]" for reference herein.

[4] The recited §120 priority claim of the Seventh Patent ('381 Patent) is not conclusive as to the correct claim of priority. "[The] inclusion of prior application information in the patent does not necessarily indicate that the claims are entitled to the benefit of the earlier filing date." MPEP §202.02

[5] A copy of the [First] Preliminary Amendment dated March 22, 2004, U.S. Pat. Appln. Ser. No. 10/717,271 (Fifth Application) is attached as Exhibit B.

Examination (RCE) filed September 2, 2008 in the Fifth Application, applicants amended the list of prior applications relied upon for §120 priority.[6] In this [Second] Preliminary Amendment:

(1) the recitations of continuity which earlier connected the Fifth Application to the First, Second, Third and Fourth Applications were deleted;

(2) the text asserting the claim to the benefit of two 1996 U.K. applications was deleted; and

(3) the First, Second, Third and Fourth Applications, and the two U.K. applications, were merely "incorporated by reference."

On October 1, 2008, applicants filed a Request for Corrected Updated Filing Receipt which attached the [Second] Preliminary Amendment filed September 2, 2008.[7] A Corrected Filing Receipt was mailed by the PTO on February 5, 2009.[8] The Receipt sets forth all of the pertinent data of the '217 Application, including its correct §120 priority claim, as follows:

> "**Domestic Priority data as claimed by applicant.**
> This appln claims benefit of 60/462,238 04/10/2003"

The §120 priority of the Fifth Application was thus limited to the Provisional Application.[9] As a result of this deliberate disclaimer of §120 priority in the Fifth Application, all applications with a filing date earlier than April 10, 2003, including the First, Second, Third and Fourth Applications, were intentionally excluded from the §120 claim for priority of the Fifth Application.

The break in the chain of priority can be seen in the following chart:

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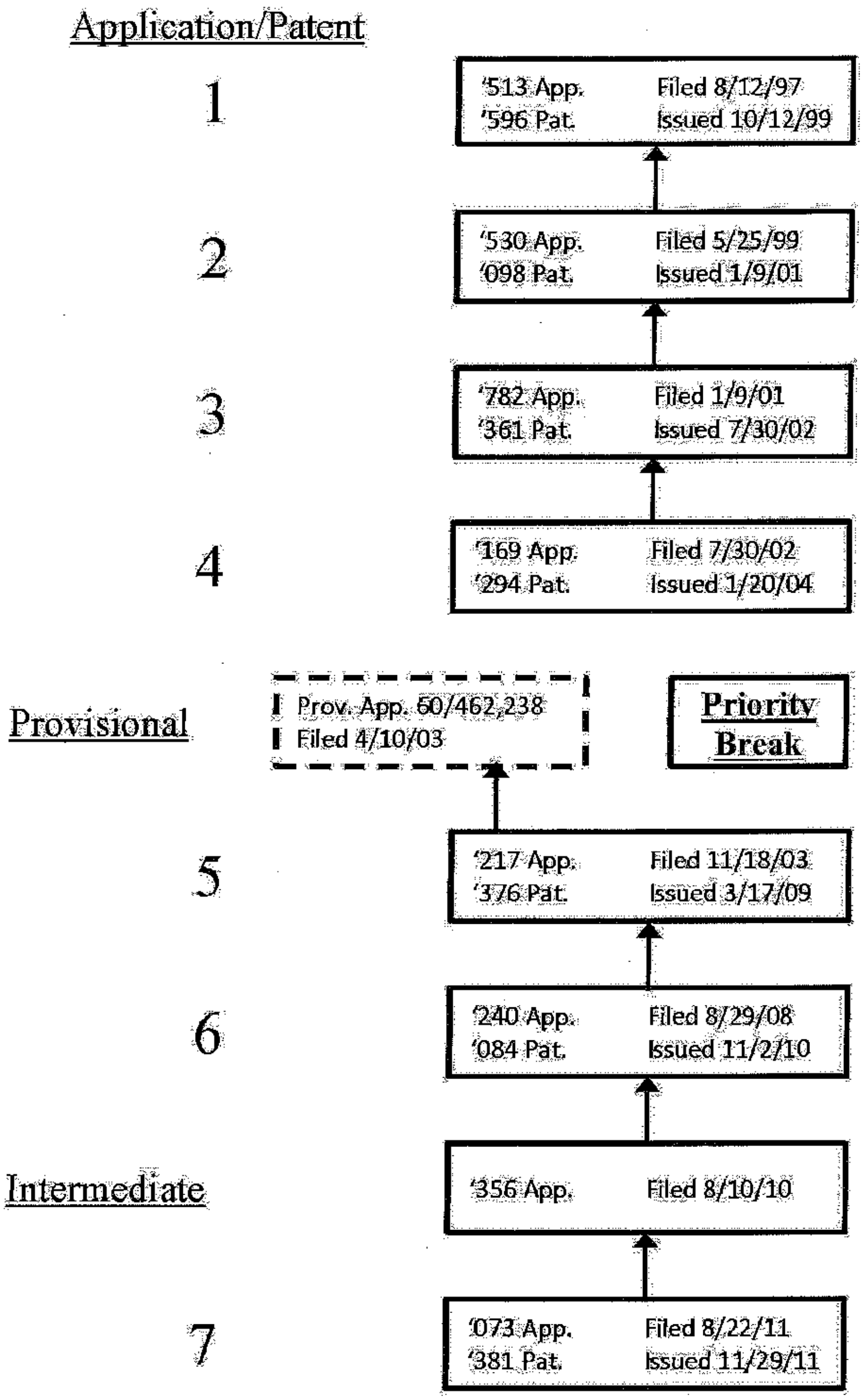
[6] A copy of the [Second] Preliminary Amendment dated September 2, 2008, U.S. Pat. Appln. Ser. No. 10/717,217, (Fifth Application) is attached as Exhibit C.

[7] A copy of the Request For Corrected Updated Filing Receipt, dated October 1, 2008, U.S. Pat. Appln. Ser. No. 10/717,217, (Fifth Application) is attached as Exhibit D.

[8] A copy of the Corrected Filing Receipt, dated February 5, 2009, U.S. Pat. Appln. Ser. No. 10/717,217, (Fifth Application) is attached as Exhibit E.

[9] A copy of the Fifth Patent, U.S. Patent No. 7,504,376, the first page of which recites the §120 priority claimed by the applicants, is attached as Exhibit F.


Application/Patent
1
'513 App. Filed 8/12/97
'596 Pat. Issued 10/12/99
2
'530 App. Filed 5/25/99
'098 Pat. Issued 1/9/01
3
'782 App. Filed 1/9/01
'361 Pat. Issued 7/30/02
4
'169 App. Filed 7/30/02
'294 Pat. Issued 1/20/04
Provisional
Prov. App. 60/462,238
Filed 4/10/03
Priority Break
5
'217 App. Filed 11/18/03
'376 Pat. Issued 3/17/09
6
'240 App. Filed 8/29/08
'084 Pat. Issued 11/2/10
Intermediate
'356 App. Filed 8/10/10
7
'073 App. Filed 8/22/11
'381 Pat. Issued 11/29/11

B. The Invention Claimed in the Fifth Application Was Not Entitled to An Earlier Priority Because It Was Not Disclosed In Any Prior Non-Provisional Application

The deletion of the priority claim in the [Second] Preliminary Amendment of the Fifth Application to the First, Second, Third and Fourth Applications was required under 35 U.S.C. §120 ("Statute") because none of the claims pending in the Fifth Application was disclosed, within the meaning of 35 U.S.C. § 112, first paragraph, in any of the First, Second, Third or Fourth Applications.

The Fifth Application was originally identified as a "continuation-in-part" ("CIP") of the Fourth Application, because the Fourth Application (as well as the Third, Second and First Applications) did not disclose compositions containing beta-alanine *and glycine,* or, beta-alanine in specific numerical dosages. The Fifth Application presented claims to compositions containing beta-alanine *and glycine,* and, beta alanine in specific numerical dosages. These claims had no §112 support in any of the First, Second, Third or Fourth Applications. Accordingly, a claim for priority in the Fifth Application to any of the First, Second, Third or Fourth Applications would have been improper as non-compliant with §120, first sentence.

The deletion of the §120 priority claim in the Fifth Application to all the prior Applications was not only required, it also provided a benefit to the applicants of adding *six years* to the term of the Fifth Patent. *See* MPEP § 201.11 III G.

C. The Claim to Priority in the Sixth Application is Defective.

The claim to §120 priority set forth in the Sixth Application and Sixth Patent is defective. The priority claim of the Sixth Application and Sixth Patent states, in part, as follows:

> Continuation of application No. 10/717,217, filed on Nov. 18, 2003, now Pat. No. 7,504,376 [Fifth Application] and a continuation-in-part of application No. 10/209,169, filed on July 30, 2002, now Pat. No. 6,680,294 [Fourth Application]. . .

This priority claim asserts that the Sixth Application is a continuation-in-part application of the Fourth Application. But the Sixth Application cannot be a continuation-in-part of the Fourth Application because the Fourth Application issued on January 20, 2004 and the Sixth Application was not filed until August 29, 2008, four and one half years later. Hence there was no "co-pendency" between the Sixth and Fourth Applications.

Nor can the priority claim quoted above be interpreted to mean that the Fifth Application is a "continuation-in-part" of the Fourth Application. As described above, in the Fifth Application, applicants deliberately cancelled the original claim of §120 priority to the First, Second, Third and Fourth Applications. Hence there is no interpretation of the §120 priority claim in the Sixth Application which can be considered correct. [10]

The Corrected Filing Receipt issued February 5, 2009 (Exhibit E) replaced and superseded the original Filing Receipt as of the date of filing of the Fifth Application and the corrected claim to priority replaced the original claim to priority as of the date of filing of the Fifth Application. The Corrected Filing Receipt, with effect retroactive to the filing date of the Fifth Application, stated the correct lineage of the Fifth Application from its filing date to its grant date and its asserted §120 priority claim only back to April 10, 2003.[11]

After applicants deleted the §120 priority claim in the Fifth Application to the four earlier Applications on October 1, 2008, and after the PTO accepted applicants' cancellation of priority to the four earlier Applications and issued the Corrected Filing Receipt on February 5, 2009,

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[10] A copy of the Sixth Patent which is Patent No. 7,825,084 is attached as Exhibit G.

[11] This analysis is consistent with the provisions of 35 U.S.C. §120, which provides, in relevant part, that any application may be entitled to claim the benefit of an earlier co-pending application if it "contains or is *amended* to contain" a specific reference to the earlier filed application. (emphasis added)

applicants had a duty to affirmatively advise the PTO of the incorrectly asserted §120 priority claims in the Sixth Application and to correct that priority.

D. The Claim to Priority in the Seventh Application is Defective

The claim to priority set forth in the Seventh Application is also defective. In the First Preliminary Amendment Under 37 C.F.R. 1.115 filed in the Seventh Application on August 22, 2011,[12] the relevant portion of the §120 priority claim of the Seventh Application stated:

> Continuation of application No. 12/806,356, filed on August 10, 2010 [Intermediate Application], which is a continuation of application No. 12/231,240, filed on Aug. 29, 2008, now Pat. No. 7,825,084 [Sixth Application], which is a continuation of application No. 10/717,217, filed on Nov. 18, 2003, now Pat. No. 7,504,376 [Fifth Application], <u>and a continuation-in-part of application No. 10,209,169, filed on Jul. 30, 2002,</u> now Pat. No. 6,680,294 [Fourth Application]. (emphasis added)

As stated above, the Sixth Application cannot be a continuation-in-part of the Fourth Application because there was no "co-pendency" between the Sixth and Fourth Applications. Nor can the Fifth Application be a continuation-in-part of the Fourth Application because applicants *cancelled* the §120 priority claim of the Fifth Application to the Fourth Application and its predecessors.

As a result of their deliberate and intentional disclaimer of priority in the Fifth Application, all continuing applications filed after the Fifth Application, including the Sixth Application and the Seventh Application (which issued as the subject Seventh Patent, the '381 Patent), have a §120 priority date no earlier than April 10, 2003. The only path in the chain of co-pending applications from the Seventh Application back to the First Application (which issued as the First Patent), is through the Fifth Application, but the Fifth Application claims

[12] A copy of the First Preliminary Amendment Under 37 C.F.R. 1.15, in U.S. Pat. Appln. Ser. No. 13/215,073 (Seventh Application) on August 22, 2011 is attached as Exhibit H.

priority no earlier than April 10, 2003. The Seventh Application cannot have a §120 priority claim earlier than the priority claim of the Fifth Application. Thus, the Seventh Application has a priority claim no earlier than April 10, 2003.

E. Rulings of the Federal Courts and of the PTO Confirm that the Priority Claim of the '381 Patent is Defective

A basic prerequisite to obtaining the benefit of an earlier application in a later application under 35 U.S.C. §120 is that the later and the earlier application must be "co-pending." This "co-pendency" requirement is in §120, which provides:

> An application for patent for an invention . . . in an application previously filed in the United States, . . . which is filed by an inventor or inventors named in the previously filed application shall have the same effect, as to such invention, as though filed on the date of the prior application, *if filed before the patenting or abandonment of or termination of proceedings on the first application or on an application similarly entitled to the benefit of the filing date of the first application* and if it contains or is amended to contain a specific reference to the earlier filed application. (emphasis added)

§120 thus does not permit the applicants to claim priority directly from the Sixth Application to the Fourth Application because the Sixth and Fourth Applications were not "co-pending."

Moreover, "co-pendency" by itself, while necessary, is not sufficient. Even where there is co-pendency between a later and an earlier application, as there was here between the Sixth and the Fifth Applications and between the Fifth and the Fourth Applications, in order to properly assert the claims, *each* application *must* assert a claim to the priority of its immediate (or another co-pending) predecessor or the §120 claim will not be recognized. *Struthers Patent Corp. v. Nestle Co.*, 558 F.Supp. 747 (D.N.J 1981); *Encyclopedia Britannica, Inc. v. Alpine Electronics of America, Inc.*, 643 F.Supp.2d 874 (W.D.Tex. 2009).

In *Struthers*, the court held that a second application in the chain (Ganiaris II) was never effectively amended prior to its termination to contain a specific reference to a first application (Ganiaris I) 558 F.Supp. 811. As a consequence, although the fourth application in the series (Ganiaris IV) properly claimed the benefit of the second application (Ganiaris II), it could not claim the benefit of the first application (Ganiaris I). The applicant's attempt in Ganiaris IV to amend Ganiaris II to refer to Ganiaris I was deemed a nullity. The court relied on *Clover Club Foods v. Gottschalk*, 178 USPQ 505 (C.D. Cal. 1973) also involving four applications, which had held:

> In a series of four patent applications, the fourth application in the series is not entitled to the benefit of the filing date of the first application in the series, if the second application in the series does not contain a *specific reference* to the first application in the series. 35 U.S.C. §120. (emphasis added).

In *Britannica*, because of certain informalities in the second of three patent applications, the second patent application was never accorded a filing date before it was abandoned, and, it did not contain the required *specific reference* to its predecessor application. The applicant argued that under the Statute its second application had been "entitled to" claim benefit and that that entitlement sufficed to permit the third application to claim back to the first application, even though the second application never made the required *specific reference* to the first application. However, the court rejected that argument. 634 F. Supp.2d 881.

Referring to and adopting two decisions of the PTO in earlier related proceedings, the court concluded that §120 requires that

> ". . . each application in the chain must individually meet the requirements of the statute, and that for any application to be entitled to the filing date of a prior-filed application, it must have been both filed before the prior-filed application and make explicit reference to the prior-filed application. See 35 U.S.C. §120. See also Clover Club Foods Co. v. Gottschalk, 178 U.S.P.Q. 505, 508

> (C.D. Cal. 1973). The Court finds no inconsistencies in the Patent Office's interpretation [**23] or application of Section 120 in this case. Because the 2nd Application contains no specific reference to the 1st Application, the Court finds that the 3rd Application is not entitled to the benefit of the filing date of the 1st Application as the 2nd Application breaks the chain of priority. 643 F. Supp. 2d 882.

The court also rejected the patentee's claim that, because the PTO permitted the third application to issue with a claim to priority through the second application and thence to the first application, the PTO had waived any requirement that the second application cross reference the first.

Here, although the Fifth Application was co-pending with the Fourth Application and applicants originally made a claim in the Fifth Application to the Fourth Application, applicants' subsequent and intentional disclaimer of priority in the Fifth Application to the First, Second, Third and Fourth Applications irrevocably broke the link between the Fifth and Fourth applications. The express and intentional disclaimer resulted in the Seventh Application not being entitled to §120 priority back to the First, Second, Third and Fourth Applications.

III. The '596 Patent Anticipates Claims 1-14 of the '381 Patent

In the following section, Requestor demonstrates that there is a reasonable likelihood that Requestor will prevail in invalidating claims 1-14 of the '381 Patent.

Because the earliest effective filing date for the '381 Patent is April 10, 2003, any patent or printed publication disclosing the invention claimed in the '381 Patent dated prior to April 10, 2002 is prior art rendering the '381 Patent invalid under 35 U.S.C. § 102(b). The First Patent (the '596 Patent) issued October 12, 1999 is therefore § 102(b) prior art to the subject Seventh Patent ('381 Patent). The Second Patent (the '098 Patent) issued January 9, 2001 on the Second Application is also §102(b) prior art to the subject Seventh Patent.

The specification of the '596 Patent is identical to the specification of the '381 Patent with respect to the subject matter claimed in the '381 Patent.

A. **The Preamble to Claim 1 of the '381 Patent is Not a Claim Limitation**

The preamble of claim 1 recites "A human dietary supplement.....". That preamble is not a limitation of a composition claim because it merely states a purpose or intended use of the composition. MPEP § 2111.02; *Pitney Bowes, Inc. v. Hewlett Packard Co.* 182 F3d 1298, 1305, 51 USPQ 2d 1161, 1165 (Fed. Cir. 1999).

Moreover, in a litigation styled, National Alternatives, Inc. v. Vital Pharmaceuticals, Inc., C.A. No. 09-626 (GMS), involving the First, Second and Third Patents, the U.S. District Court for the District of Delaware construed claims having a virtually identical preamble to that of claim 1 of the '381 Patent and found that the preamble "dietary supplement" was not a claim limitation. In a Markman Order dated May 31, 2011, Chief Judge Sleet stated:[13]

> After having considered the submissions of the parties and hearing oral argument on the matter, IT IS HEREBY ORDERED, ADJUDGED, and DECREED THAT, as used in the asserted claims of U.S. Patent Nos. 5,965,596 (the "'596 patent"); 6,172,098 (the "'098 patent"); and 6,426,361 (the "'361 patent")
>
> 1. The term "dietary supplement" will not be construed because it is not a limitation.[2]
>
> [2] A threshold issue is whether a term located in a claim's preamble is a limitation that must be construed. "In general, a preamble limits the invention if it recites essential structure or steps, or if it is 'necessary to give life, meaning, and vitality to the claim. Conversely, a preamble is not limiting 'where a patentee defines a structurally complete invention in the claim body and uses the preamble only to state a purpose or intended use for the invention.'" *Catalina Mktg. Int'l v. Coolsavings.com, Inc.*, 289 F. 3d 801, 808 (Fed. Cir. 2002) (internal citations omitted). Here, the court finds that that the term "dietary supplement," which only appears in the preamble of various claims, is not a limitation

[13] A copy of the Markman Order dated May 31, 2011 is attached as Exhibit I.

> requiring construction. Interestingly, this term appears in the preambles of claims in which the body contains the same exact language as the body of claims that have "composition" in the preamble. (See, e.g., '361 patent, claims 1 and 5; claims 10 and 17; and claims 22 and 27.) At first glance this may make it seem like the term must be a limitation. On closer examination, however, the principles articulated by the Federal Circuit are not satisfied. In particular, there is no evidence that the patentees used this term to distinguish prior art. *See Catalina Mktg. Int'l*, 289 F.3d at 808 ("[C]lear reliance on the preamble during prosecution to distinguish the claimed invention from the prior art transforms the preamble into a claim limitation because such reliance indicates use of the preamble to define, in part, the claimed invention."). Also, the specification does not demonstrate that the term was a necessary and defining aspect of the invention. *See On Demand Mach. Corp. v. Ingram Indus.*, 442 F.3d 1331, 1343 (Fed. Cir. 2006) ("In considering whether a preamble limits a claim, the preamble is analyzed to ascertain whether it states a necessary and defining aspect of the invention, or is simply an introduction to the general field of the claim."). In fact, the specification simply notes that the claimed "composition *can be* a dietary supplement." ('361 patent, col. 3, 1.41 (emphasis added).) For these reasons, the court agrees with the defendants that the term "dietary supplement" is not a claim limitation requiring construction.

When the preamble of claim 1 is properly ignored because it is not a claim limitation, the claim merely recites beta-alanine in the form of a single amino acid. Beta-alanine is a common, naturally-occurring amino acid. Its structure, properties and uses have been disclosed in countless prior art references and it has been available for many decades. See for example, U.S. Patents Nos. 3,932,501 (1976) and 4,886,889 (1989).

The '381 Patent, like its earlier predecessors, does not disclose the source of the beta-alanine employed in the Examples, presumably because the applicants recognized that beta-alanine is known to be available. Were that not the case, the specification would be wanting under 35 U.S.C. §112, first paragraph.

Thus, *any* prior art composition containing beta-alanine, even if *not* disclosed for use as a "human dietary supplement," is anticipating prior art to claims of the '381 Patent, because "human dietary supplement" is only in the claim preamble.

B. <u>Even If the Claim Preamble is Accorded Patentable Weight, the '596 Patent Discloses Use of the Composition of Claim 1 as a Dietary Supplement</u>

Even if the claim preamble term "human dietary supplement" is accorded patentable weight, the '596 Patent is replete with disclosures of human dietary supplements containing beta-alanine. See, e.g., '596 Patent col. 5, line 46, "The composition can be a dietary supplement," and col. 5, lines 52-53 which refer to use of the composition by "sportsman, athletes, body-builders, . . . "

C. <u>Claim 1 of the '381 Patent Uses "Comprising" and Hence is Open Ended</u>

Claim 1 of the '381 Patent is open-ended. Claim 1 recites:

1. A human dietary supplement

comprising at least one of: an amino acid ....[which] is beta-alanine; ...
an ester of beta-alanine ...;
or an amide of beta-alanine....

Because the claim uses "comprising" language, a prior art disclosure of a composition having any one of the recited components will anticipate the claimed composition, even if the disclosed composition in the prior art contains additional components. Thus, the prior art will invalidate the claim, if it discloses only *one* of (i) beta-alanine, (ii) an ester of beta-alanine or (iii) an amide of beta-alanine, which is not part of a dipeptide, polypeptide or oligopeptide.

Virtually all of the specification of the '596 Patent anticipates claim 1. Attached is a claim chart showing some of the anticipating passages.

'381 Patent Claims	'596 Patent Disclosure
1. A human dietary supplement	"The composition can be a dietary supplement" (col. 5, line 46); and can be used by "sportsmen, athletes, bodybuilders..." (col. 5, lines 52-53).
comprising at least one of: an amino acid wherein said amino acid is beta-alanine that is not part of a dipeptide, polypeptide or oligopeptide; an ester of beta-alanine that is not part of a dipeptide, polypeptide or oligopeptide; or an amide of beta-alanine that is not part of a dipeptide, polypeptide or oligopeptide.	"The compositions include mixtures of creatine and beta-alanine, creatine, beta-alanine and L-histidine, or creatine and active derivatives of beta-alanine or L-histidine. Each of the beta-alanine or L-histidine can be the individual amino acids, or components of dipeptides, oligopeptides, or polypeptides." (col. 2, lines 30-35)[14] "In another aspect, the invention features a composition consisting essentially of a peptide source including beta-alanine, between about 39 and about 99 percent by weight of a carbohydrate, and up to about 60 percent by weight of water." (col. 3, lines 9-13) Additional passages which anticipate claim 1 include: col. 2, lines 42-49; col. 2, lines 53-56; col. 3, lines 1-4; col.3, lines 9-13; col. 3, lines 31-32; col. 5, lines 36-42; col. 5, lines 46-50; col. 6, lines 1-21; and all the Examples, including Example 1 which exemplified the "invention" in a test involving horses.
2. The human dietary supplement of claim 1, further comprising a carbohydrate.	"The composition can include carbohydrates (e.g., simple carbohydrates), insulin, or agents that stimulate the production of insulin." (col.

[14] In relying on the quoted passage as an anticipation of the claim language, Requestor relies on the statement that the "...beta-alanine ... can be the individual amino acids. ..." but does not concede that the quoted text supports the negative limitation in claim 1 "... that is not part of a dipeptide, polypeptide or oligopeptide." That negative limitation in the claims appeared for the first time in the claims of the Seventh Application and has no corresponding written description in the specification of the '596 Patent or '381 Patent and hence the claims of the '381 Patent do not comply with the written description requirement of 35 U.S.C. §112, first paragraph. See MPEP 2173.05 (i).

	5, lines 43-45)
3. The human dietary supplement of claim 2, wherein the carbohydrate is a simple carbohydrate.	"The composition can include carbohydrates (e.g., simple carbohydrates), insulin, or agents that stimulate the production of insulin." (col. 5, lines 43-45)
4. The human dietary supplement of claim 3, wherein the simple carbohydrate is glucose.	"The composition can include carbohydrates (e.g., simple carbohydrates), insulin, or agents that stimulate the production of insulin." (col. 5, lines 43-45)
5. The human dietary supplement of claim 1, further comprising L-Histidine.	"The blood plasma concentrations of beta-alanine, L-histidine and creatine can be increased by ingestine or infusion of beta-alanine, L-histidine, and creatine, or active derivatives thereof." (col. 5, lines 36-39)
6. The human dietary supplement of claim 1, further comprising insulin.	"The composition can include carbohydrates (e.g., simple carbohydrates), insulin, or agents that stimulate the production of insulin." (col. 5, lines 43-45)
7. The human dietary supplement of claim 1, further comprising an insulin stimulating agent.	"The composition can include carbohydrates (e.g., simple carbohydrates), insulin, or agents that stimulate the production of insulin." (col. 5, lines 43-45)
8. The human dietary supplement of claim 1, further comprising creatine.	"The creatine (e.g., creatine monohydrate) dosage can be between about 5 milligrams to 200 milligrams per kilogram body weight." (col 5, lines 49-51)
9. The human dietary supplement of claim 8, wherein the creatine is creatine monohydrate.	"The creatine (e.g., creatine monohydrate) dosage can be between about 5 milligrams to 200 milligrams per kilogram body weight." (col 5, lines 49-51)
10. The human dietary supplement of claim 1, wherein the dietary supplement is in liquid form.	"The composition can be in solid form or liquid form or the form of a suspension which is ingested, or in liquid form or suspension for infusion into the body." (col. 5, lines 60-63)

11. The human dietary supplement of claim 1, wherein the dietary supplement is in solid form.	"The composition can be in solid form or liquid form or the form of a suspension which is ingested, or in liquid form or suspension for infusion into the body." (col. 5, lines 60-63)
12. The human dietary supplement of claim 1, wherein the dietary supplement is in ingestible suspension.	"The composition can be in solid form or liquid form or the form of a suspension which is ingested, or in liquid form or suspension for infusion into the body." (col. 5, lines 60-63)
13. The human dietary supplement of claim 1, wherein the human dietary supplement is effective in delaying the onset of fatigue in a human.	"The methods and compositions can be used to increase beta-alanylhistidine dipeptide by, for example, sportsmen, athletes, body-builders, synchronized swimmers, soldiers, elderly people, . . . to avoid or delay the onset of muscular fatigue." (col. 3, lines 51-56)
14. The human dietary supplement of claim 1, wherein the human dietary supplement is effective in protecting the function of a creatine-phosphorylcreatine system.	"During sustained intensive exercise, or exercise sustained under conditions of local hypoxia, the accumulation of hydronium ions formed during glycolsis and the accumulation of lactate (anaerobic metabolism) can severely reduce the intracellular pH. The reduced pH can compromise the function of the creatine-phosphorylcreatine system." (col. 1, lines 50-55) "The method of claim 3, wherein increasing the amount of creatine in the human tissue includes providing an amount of creatine to the blood or blood plasma effective to increase the concentration of creatine in the human tissue." (claim 5, '596 Patent) "An increase in the muscle content of creatine and beta-alanylhistidine dipeptides can increase the tolerance of the cells to the increase in hydronium ion production with anaerobic work, and to lead to an increase in the duration of the exercise before the onset of

	fatigue." (col. 3, lines 41-45) Thus, increasing creatine protects the function of the creatine-phosphorylcreatine system.

IV. Claims 1-14 Are Anticipated or Obvious from the Prior Art

A. The Composition of Claim 1 of the '381 Patent Is Anticipated by Each of Several Prior Art References

The anticipating references are:

1. Asatoor, et al., Intestinal absorption of carnosine and its constituent amino acids in man, Gut, 1970, Vol. II, pp. 250-254 ("Asatoor"). (Exhibit J).

2. European Patent Office Publication No. 0 280 593 B1, June 12, 1991. ("EP '593") including both the text in French and an English language translation provided by the EPO. (Exhibit K).

3. Gardner, et al., Intestinal Absorption of the Intact Peptide Carnosine in Man, and Comparison with Intestinal Permeability to Lactulose, Journal of Physiology (1991), Vol. 439, pp. 411-422 ("Gardner"). (Exhibit L).

4. De Lacharriere, et al., U.S. Patent No. 5,869,068, Feb. 9, 1999 ("De Lacharriere '068 Patent") and De Lacharriere et al., U.S Patent No. 5,976,559, November 1, 1999 ("De Lacharriere '559 Patent"). (Exhibit M).

5. Wu, et al., Proximate Composition, Free Amino Acids and Peptides Contents in Commercial Chicken and Other Meat Essences, Journal of Food and Drug Analysis, (2012) Vol. 10, No. 3, 2002, pp. 170-177 ("Wu"). (Exhibit N).

6. Li, et al., Bioactivities of Chicken Essence, Journal of Food Science, 2012, 77: R105-R110. ("Li"). (Exhibit O)

Each of the references is discussed below.

(1) Claim 1 is Anticipated By Asatoor

Claim 1 is anticipated under 35 U.S.C. § 102(b) by Asatoor (1970).

Asatoor discloses experiments in which five human test subjects ingested a mixture of beta-alanine and L-histidine.

> "*Five normal adults were investigated, each individual ingesting carnosine and equivalent amounts of a mixture of β-alanine and L-histidine after an overnight fast.* The two tolerance tests in each subject were carried out at intervals of at least two weeks. Dosage of carnosine was 0.286 m mole/kg body weight, corresponding to 20 m mole per standard 70 kg male. Histidine and ß-alanine were taken together [by the adult subjects] in an amount . . . Both the dipeptide and the amino acid mixture were taken dissolved in 500 ml water." (p 250-251) (italics added)

Asatoor thus discloses a human dietary supplement comprising beta-alanine that is not part of a dipeptide, polypeptide or oligopeptide, according to the '381 Patent, claim 1.

(2) Claim 1 is Anticipated By EP '593

Claim 1 is anticipated under 35 U.S. C. § 102(b) by EP '593 (1991).

EP '593 discloses human dietary supplements containing beta-alanine that are not part of a dipeptide, polypeptide or oligopeptide, according to the '381 Patent, claim 1. As such they anticipate claim 1 of the '381 Patent under 35 U.S.C. § 102 (b).

The following text from the English language translation is relevant.

> [0012] The present invention thus provides a *new composition based on [beta]-alanine* and vitamins, used to treat cancer.
>
> [0013] The new composition in accordance with the present invention comprises at least one vitamin and [beta]-alanine represented by general formula (1):
> H2N – (CH2) 2 – COOH (1)
> or a salt thereof acid or base.
>
> [0014] More particularly, the composition according to the invention comprises the [beta]-alanine of the formula (1) above and one or more vitamins selected from vitamin A, vitamin B, vitamin B2, vitamin Bs, pyridoxine, ascorbic acid, tocopherol, vitamin D2 and vitamin PP.

> [0015] *[beta]-alanine can be used individually* or, where appropriate, in combination with one or more other amino acids, for example 5-alanine and glycine, or [beta]-alanine, and taurine, can be combined.
>
> [0016] In addition to vitamins and the amino acid indicated above, the composition may advantageously also contain additives such as glucose, [beta]-carotene and other vitamins such as biotin and vitamin B12.
>
> [0020] The components constituting the composition of the present invention can be administered in combination or separately, preferably orally.
>
> It is for example the amino acid mix, vitamins and, where appropriate, glucose and other additives in the form of a powder which can be packaged in various forms usual, for example in sachets, capsules or capsules. (italics added)

The Examples (seen in the French text but not in the English translation) also anticipate claim 1. The composition described in Example 1 is set forth below.

[0026] A mixture of the following components below.

ß-alanine	*90 g*
taurine	10 g
Glucose	30 g
Vitamine A	60.000 UI
Vitamine B_1	24 mg
Vitamine B_2	18 mg
Vitamine B_5	48 mg
Pyridoxine (chlorhydrate)	24 mg
Acide ascorbique	600 mg
Vitamine D_2	12.000UI
Tocophérol (acétate)	24 mg
Vitamine PP	120 mg
ß-carothéne	100 mg

(italics added)

EP '593 thus discloses a human dietary supplement comprising beta-alanine that is not part of a dipeptide, polypeptide or oligopeptide, according to the '381 Patent, claim 1.

<u>(3) Claim 1 is Anticipated By Gardner</u>

Claim 1 is anticipated under 35 U.S. C. § 102(b) by Gardner (1991).

Gardner discloses an experiment in which a human subject ingested a "test meal" comprising beta-alanine and histidine in syrup.

> <u>"Experimental Procedure</u>
> Subjects ingested a test meal which was designated (a) 'blank', (b) 'isotonic' (nominally) . . . . All subjects consumed at least one of each test meal . ... The 'blank' meal comprised 92.5 ml hot water, 7.5 ml Duphulac syrup . . . and 1 g ramnose . . . . The Duphulac contained 5 g lactulose and traces of galactose and lactose in aqueous solution. The 'isotonic' test meal contained the same ingredients with the addition of 4 g carnosine . . .. *(In an additional experiment on one subject, an approximately isotonic test meal containing 2 g ß-alanine plus 2 g histidine instead of carnosine was taken).* (pp 412-413) (italics added)

Gardner thus discloses a human dietary supplement comprising beta-alanine that is not part of a dipeptide, polypeptide or oligopeptide, according to the '381 Patent, claim 1.

<u>(4) Claim 1 is Anticipated By De Lacharriere '068 and '559</u>

Claim 1 of the '381 Patent is anticipated by both De Lacharriere '559 and De Lacharriere '068 under 35 U.S.C. § 102(b) because their issue dates are more than one year before the earliest possible effective filing date of the '381 Patent, April 10, 2003. Claim 1 is anticipated by De Lacharriere '068 and '559 under 35 U.S.C. §102 (e) even if the '381 Patent were to be accorded the purported priority date of Aug. 12, 1997.

The '559 and '068 Patents have identical specifications and disclose beta-alanine in a composition. For example De Lacharriere '559 Patent states:

> Mention may be made, as agonist substances, which can be used in the present invention for activating glycine-strychnine sensitive receptors, of ...*ß-alanine* ... (col. 4, lines 3-5) (italics added)

Even though the compositions are topical skin cream compositions, these disclosures anticipate claim 1 because the preamble of claim 1 is not a limitation.

The PTO rejected claims 44 and 45 of the Third Application in a Final Office Action on August 15, 2001[15] as not novel and as obvious and thus failing to comply with 35 U.S.C. §120(e) in view of De Lacharriere '559. The rejected claims were virtually identical to claim 1 of the '381 Patent. The rejected claims recited:

> 44. A human dietary supplement comprising beta-alanine.
>
> 45. A human dietary supplement comprising an amino acid or an active derivative thereof selected from the group consisting of a beta-alanine, an ester of beta-alanine and an amide of a beta-alanine.

Applicants cancelled claims 44 and 45 without disputing the PTO's final rejection of these claims over De Lacharriere '559, thereby conceding that this reference disclosed the subject matter of finally rejected claims 44 and 45.

While Applicants filed an IDS in the Seventh Application listing De Lacharriere '559, this reference was merely listed among the 118 references and applicants did not bring this reference specifically to the Examiner's attention, even though applicants had conceded that the similarly worded claims 44 and 45 were unpatentable over this reference ten years earlier in the Third Application. The Examiner did not apply De Lacharriere '559 Patent to any of the claims of the subject Seventh Application. Indeed, there was no substantive Office Action in the subject Seventh Application.

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[15] A copy of the Final Rejection dated August 15, 2001, U.S. Pat. Appln. Ser. No. 09/757,782 (Third Application) is attached as Exhibit P.

Thus, the DeLacharriere '559 Patent anticipates Claim 1 of the '381 Patent, a finding made by the PTO ten years earlier.

(5) Claim 1 is Anticipated By Wu and Li

Wu (April 11, 2002) discloses that chicken, beef and fish *extracts* commonly consumed in Southeast Asia contain beta-alanine.

> In Southeast Asia region, particularly in Chinese communities, chicken essence is consumed as a traditional health food for several ailments, including the use as a nutritional supplement (p. 170)
>
> The present study was therefore undertaken to establish basic data of proximate composition, FAA, dipeptides and other small peptides in chicken, beef, clam and eel essences available in the markets. (p. 170)
>
> Commercial meat essences, including beef, freshwater clam, hard clam, eel and six types of chicken essence, were purchased from June 1999 to December 2000 from markets in Keelung and Taipei, Taiwan. (p. 170)
>
> Table 2 (p. 172) and Table 3 (p. 173) disclose that *the essences contain beta-alanine.* Table 4 (p. 173) discloses that the essences contain carnosine.

These analyses demonstrate that commercial chicken essences, available in this country as well as Southeast Asia for scores of years, contain a spectrum of amino acids, including beta-alanine as well as carnosine. Wu thus discloses a dietary supplement comprising beta-alanine that is not part of a dipeptide, polypeptide or oligopeptide, according to the '381 Patent, claim 1.

Confirming the centuries-old availability of chicken essences, Li, referring to Geissler et al. (1996) and other references, discusses the preparation of Brand's Essence of Chicken -- a product that has been around for more than 170 years -- by a water extraction process.

> For example, Brand's Essence of Chicken (BEC), which has a history more than 170 y, is produced via a water extraction process from chicken meat for several hours

> under high-temperature, followed by centrifugation to remove fat and cholesterol, vacuum concentration to 3- to 4- fold, sterilization by high temperature and pressure before bottling. This kind of chicken essence is rich in protein, and low in sugar and fat, conveniently available and consumable, and easy for household storage. Previous studies confirmed that BEC not only contains indispensable amino acids including threonine, valine, methionine, isoleucine, leucine, phenylalanine, lysine, and tryptophan, but also contains a substantial proportion of free amino acids such as histidine and arginine (shown in Table 1) (Geissler and other 1996; Zain and Jamalulail 2003). Besides many minerals, trace elements, vitamins, and some special nutrients such anserine, carnosine, and taurine are also detected in BEC.

Thus chicken essence, a product more than 170 years old, is a composition containing the full complement of amino acids, including beta-alanine.[16]

Accordingly, a composition comprising beta-alanine not in the form of a peptide, polypeptide or oligopeptide according to claim 1 of the '381 Patent is anticipated by chicken essence products available since the mid-1800s.

B. The Composition of Claim 1 of the '381 Patent is Obvious from the Prior Art

The following additional references are relied on in support of the obviousness of claim 1:

1. European Patent Application Publication No. 0 449 787 A2 (March 19, 1991) (Applicant: Setra S.r.L) ("Setra") (Exhibit Q).

2. Bauer, et al., Biosynthesis of carnosine and related peptides by skeletal muscle cells in primary culture, Eur. J. Biochem. Vol. 219, pp. 43-47 (1994) ("Bauer") (Exhibit R).

3. Bakardjiev, et al., Transport of ß-Alanine and biosynthesis of carnosine by skeletal muscle cells in primary culture, Eur. J. Biochem. 225, pp. 617-623 (1994) ("Bakardjiev") (Exhibit S).

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[16] That chicken includes beta-alanine is acknowledged in the '381 Patent in col. 14, lines 26-43, discussing preparation of chicken broth which includes beta-alanine.

4. Casey, et al., Creatine ingestion favorably affects performance and muscle metabolism during maximal exercise in humans, Am. J. Physiol. 271 (Endocrinol. Metab. 34): E31-E37, 1996. ("Casey") (Exhibit T).

5. Reaction rate tends to increase with concentration – phenomenon explained by collision theory, Chemical kinetics, Wikipedia.org ("Wikipedia") (Exhibit U).

6. Biolo, et al., Insulin action on protein metabolism, Baillière's Clinical Endocrinology and Metabolism, Vol. 7, No. 4, October 1993 ("Biolo") (Exhibit V).

(1) Setra

Setra discloses pharmaceutical, dietetic or veterinary compositions containing carnosine or peptides related thereto as the active ingredient. In particular, Setra discusses the known beneficial properties of these compositions when they are ingested. The compositions are said to secure the known benefits of carnosine (a dipeptide, namely ß-alanyl-L-histidine, formed from the amino acids ß-alanine and L-histidine, p. 2, lines 4-5), including carnosine's ability to prevent uncontrolled proton release in the cell and thus a drop in cellular pH and to thus prevent "metabolic acidosis" and its cousequences, i.e. muscular fatigue and weakness (p. 2, lines 3-13).

The following passages are particularly relevant.

> "Therefore, according to the invention, oral pharmaceutical, dietetic or veterinary compositions containing a dipeptide selected from the group of carnosine, homocarnosine, anserine, homoanserine or ophidine or physiologically equivalent derivatives thereof such as salts, acetyl derivatives and the like are provided. Said compositions can further contain other active ingredients with complementary or anyway useful activities such as creatine, carnitine, acetylcarnitine, essential or non essential amino acids, sugars, mineral salts and vitamins. Carnosine is the preferred dipeptide." (p2, lines 28-34)
>
> "EXAMPLE 1
> Composition, to be orally administered from four times to twice a day as support to a strong muscular activity, containing carnosine, essential amino acids and sugars, in the following proportions:
> Carnosine 1.0g

Histidine	1.0g
Carnitine	0.5g
Creatine	2.0g
Glucose	1.0g
Flavouring agents	(p2, lines 49-58)

EXAMPLE 2

Composition, to be administered to subjects with nutritional or liver metabolic deficiency or in case of muscular fatigue from three times to once a day, containing carnosine and essential amino acids in the following proportions:

Carnosine	0.35g
Creatine	0.75g
Histidine	0.35g
Carnitine	0.175g
Flavouring agents"	(p3, lines 1-10)

(2) **Claim 1 is Obvious Over Setra in View of Asatoor**

The composition of claim 1 is obvious over Setra in view of Asatoor. Setra discloses that the compositions may include, *inter alia*, essential and non-essential amino acids.

> Said compositions can further contain other active ingredients with complementary or anyway useful activities such as creatine, carnitine, acetylcarnitine, essential or non essential amino acids, sugars, mineral salts and vitamins. Carnosine is the preferred dipeptide. (p 2, lines 31-34)

Asatoor in the Summary on page 250 and on page 252, first column and in Fig. 3 reports the rapid (relative to carnosine) absorption of both ß-alanine and histidine into serum.

> Serum concentrations of *ß-alanine* and L-histidine are compared in five normal adults after ingestion of the dipeptide carnosine (ß-alanyl-L-histidine) and after equivalent amounts of the constituent free amino acids. The results indicate that absorption is significantly more rapid after the ingestion of the amino acids than after the dipeptide. (p 250)
>
> It can be concluded that absorption of both *ß-alanine* and of histidine is significantly more rapid after ingestion of the free amino acids than after ingestion of the equivalent amount of carnosine. (p 252) (italics added)

It would therefore have been obvious to one skilled in the art to include beta-alanine, a known non-essential amino acid and a precursor of carnosine, alone or together with histidine in compositions as disclosed in Examples 1 and 2 of Setra because Asatoor reported rapid uptake of these individual amino acids in blood serum. Setra invites the skilled artisan to include single amino acids in his compositions and Asatoor nominates beta-alanine as the amino acid of choice. At the very least, it would have been obvious to try using beta-alanine in the Setra composition rendering Claim 1 obvious.

(3) <u>Claim 1 is Obvious Over Setra in View of Gardner</u>

The composition of claim 1 is obvious over Setra in view of Gardner. Setra, at p 2, lines 31 - 34 discloses that the compositions may include, *inter alia*, essential and non-essential amino acids, and, in Examples 1 and 2 discloses compositions containing histidine, a component of carnosine (beta-alanyl-L-histidine).

Gardner describes tests in which beta-alanine in an isotonic medium was fed to a human subject.

It would therefore have been obvious add beta-alanine to the composition of Examples 1 or 2 of Setra, rendering claim 1 obvious.

(4) <u>Claim 1 is Obvious Over Setra in View of Gardner and Asatoor</u>

The composition of claim 1 would have been obvious over Setra in view of Asatoor and Gardner for the reasons given above in sections (2) and (3).

(5) <u>Claim 1 is Obvious Setra in View of Bauer or Bakardjiev</u>

Bauer discloses in the Summary:

> Synthesis of carnosine (ß-alanyl-L-histidine) . . . could be demonstrated in primary muscle cell cultures. . . . [a] rapid increase in *ß-alanine* uptake . . . could be observed. . . .

> These results demonstrate that carnosine. . . . [is] actively synthesized by muscle cells in culture. (p. 43) (italics added)

It would have been obvious to combine Setra with Bauer and include beta-alanine as a component of a composition such as those described and exemplified in Setra, rendering claim 1 obvious.

In would likewise have been obvious to combine Setra with Bakardjiev who likewise reported synthesis of carnosine from beta-alanine by primary cultures of chicken pectoral muscles, rendering claim 1 obvious.

(6) <u>Claim 1 is Obvious Over Setra and the State of the Art Including Wikipedia</u>

The composition of claim 1 is obvious from Setra in view of the state of the art whether in 1980, 1996 or 2003.

In the preceding sections, the composition of claim 1 is demonstrated to be obvious over Setra in view of Asatoor; Setra in view of Gardner; and Setra in view of Bauer or Bakardjiev. These combinations of references demonstrate the state of the art well before 1996. There is still further evidence of obviousness.

It was applicants' objective to increase the amount of carnosine in muscle cells. Carnosine, a dipeptide (ß-alanine-L-histidine) was first isolated in 1900. The structure of carnosine and its synthesis from precursors ß-alanine and L-histidine has been known for nearly 100 years.[17] Also known for a century or more is that increasing the concentration of the reactants in a reaction will tend to drive the reaction to completion.[18]

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[17] See Bauer, p. 43, first column and footnotes 1, 2, 3.

[18] See Wikipedia

In order to increase the amount of carnosine in muscle cells, it would have been obvious to one skilled in the art to increase the amount of the carnosine precursors ß-alanine and/or L-histidine to drive the reaction and make carnosine. Given the reports of Asatoor, Gardner, Bauer and Bakardjiev, this would have been thought by the skilled artisan as likely to succeed in increasing the amount of carnosine in the blood serum and ultimately in the muscle cells. Thus it would have been *prima facie* obvious to prepare a composition of claim 1 to induce the synthesis of carnosine by muscle cells.

A skilled artisan motivated by the desire to induce the muscle cells to synthesize carnosine, knowing that beta-alanine is absorbed more rapidly into blood serum than carnosine and knowing that beta-alanine induces muscle cells to synthesize carnosine, would have prepared a composition comprising beta-alanine or one of its esters or one of its amides. Because prior art demonstrates that beta-alanine, the known precursor of carnosine is shown to be taken up in cells and to produce carnosine, it would have been obvious to one skilled in the art, given the objectives stated in the '381 Patent to prepare the composition of claim 1.

The Supreme Court has set forth the standards by which unobviousness shall be determined:

> [A] patent claim is only proved obvious if some motivation or suggestion to combine the prior art teachings can be found in the prior art, the nature of the problem, or the knowledge of a person of ordinary skill in the art. *KSR Intl. Co. v. Telefelex Inc.*, 127 S. Ct. 1727,1735, 82 USPQ 2d 1385 (2007).

Thus, ". . . a combination of *familiar elements* according to known methods is likely to be obvious when it does no more than yield predictable results." *Id.* at 1395 (emphasis added).

Here, the properties of beta-alanine were known and the desirability of increasing the rate of formation of carnosine was also known. Setra specifically invites the skilled artisan to include

non-essential amino acids in its carnosine-containing compositions. Given this disclosure in Setra, the claimed invention would have been obvious to the ordinary artisan. At the very least, it would have been obvious to try including beta-alanine in the compositions of Setra. Under KSR, that which is "obvious to try" is "obvious." It would have been obvious to the ordinary artisan motivated by the desire to increase the amount of carnosine to try introducing beta-alanine.

C. The Compositions of Claims 2, 3, 4, 5, 7, 8, 10, 11, 12, 13 and 14 are Obvious Over Setra In View of Each of Asatoor, Gardner, Bauer, Bakardjiev, and the State of the Art Including Wikipedia; the Compositions of Claim 6 Are Obvious Over Each of These Combinations of References Further In View of Biolo; and the Compositions of Claim 9 Are Obvious Over Each of These Combinations of References Further in View of Casey

Claims 2-4 of the '381 Patent recite:

> 2. The human dietary supplement of claim 1, further comprising a carbohydrate.
>
> 3. The human dietary supplement of claim 2, wherein the carbohydrate is a simple carbohydrate.
>
> 4. The human dietary supplement of claim 3, wherein the simple carbohydrate is glucose.

Claims 2, 3 and 4 are obvious over Setra in view of each of the secondary references alone, as discussed in each of Sections B(2) – B (6). Thus the composition of claim 1 is obvious over each of the five combinations of references. Claims 2, 3 and 4 are obvious because the composition of Example 2 of Setra includes and the compositions of Examples 1 and 3 of EP '593 include glucose, a simple carbohydrate.

`Claim 5 of the '381 Patent recites:

> 5. The human dietary supplement of claim 1, further comprising L-Histidine.

Claim 5 is obvious over Setra in view of each of the secondary references alone, as discussed in each of Sections B(2) – B(6), and, in particular, because the compositions of Examples 1, 2 and 4 of Setra include histidine.

Claims 6 and 7 of the '381 Patent recite:

> 6. The human dietary supplement of claim 1, further comprising insulin.

Claim 6 is obvious over Setra in view of each of the secondary references alone, as discussed in each of Sections B(2) – B(6), and, further in view of Biolo which teaches the value of insulin in dietary supplements.

> 7. The human dietary supplement of claim 1, further comprising an insulin stimulating agent.

Glucose is the most common insulin stimulating agent. Hence, the composition of claim 7 is obvious over Setra and each of the secondary references alone, as discussed in each of Sections B(2) – B (6), and, in particular, because the composition of Example 2 of Setra and the compositions of Examples 1 and 2 of EP '593 include glucose.

Claims 8 and 9 of the '381 Patent recite:

> 8. The human dietary supplement of claim 1, further comprising creatine.
>
> 9. The human dietary supplement of claim 8, wherein the creatine is creatine monohydrate.

Claims 8 and 9 are obvious over Setra in view of each of the secondary references alone, as discussed in each of Sections B(2) – B(6), and, in particular, because the compositions of

Examples 1 and 2 of Setra include creatine. Creatine monohydrate is a common form of creatine.[19]

Claims 10 – 12 of the '381 Patent recite:

> 10. The human dietary supplement of claim 1, wherein the dietary supplement is in liquid form.
>
> 11. The human dietary supplement of claim 1, wherein the dietary supplement is in solid form.
>
> 12. The human dietary supplement of claim 1, wherein the dietary supplement is in ingestible suspension.

The compositions of claims 10, 11 and 12 are all obvious from Setra in view of each of the secondary references alone, as discussed in each of Sections B(2) – B(6) above. Setra specifically teaches that

> Examples of formulations include single-dose sachets containing powders or granulates which can optionally be effervescent and can be *dissolved in water* or other beverages before use; *tablets*; soft or hard capsules; syrups; sweets and the like; containing not only carnosine and optionally other active ingredients, but also appropriate excipients such as flavouring, sweetening, effervescence agents and all those additives which are well-known to the man skilled in the art. (p2, lines 42-46) (italics added)

Claims 13 – 14 of the '381 Patent recite:

> 13. The human dietary supplement of claim 1, wherein the human dietary supplement is effective in delaying the onset of fatigue in a human.
>
> 14. The human dietary supplement of claim 1, wherein the human dietary supplement is effective is protecting the function of a creatine-phosphorylcreatine system.

---

[19] See Casey

Claims 13 and 14 are improper claims because they merely state the intended and inherent functions and properties of the claimed composition, not some specific embodiment thereof.

In any case, the prior art compositions, including the compositions described in Setra in view of each of the secondary references discussed in each of Sections B(2)- B(6) above, all will have those inherent functions. See in particular Setra, page 2, lines 11-13.

Conclusion

In view of the foregoing, there is a reasonable likelihood that Requestor will prevail with respect to each claim of the '381 Patent. Requestor respectfully requests that reexamination of the '381 Patent be ordered.

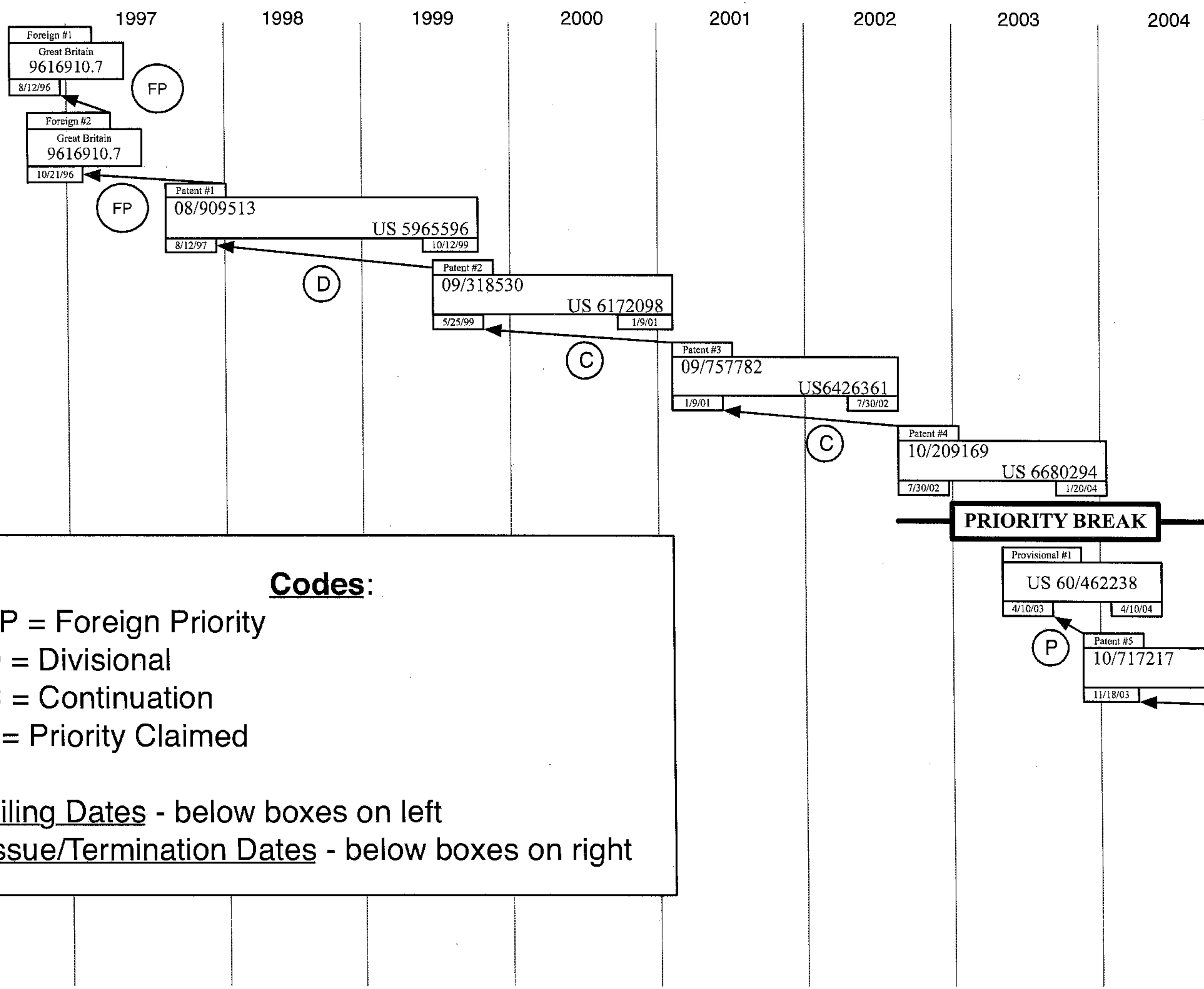
Dated: May 31, 2012

Respectfully submitted,

LUCAS & MERCANTI, LLP
Attorneys for Requester

/Barry Evans/
Barry Evans, Reg. No. 22,802
Peter J. Phillips, Reg. No. 29,691
475 Park Avenue South
New York, New York 10016
Tel.: (212) 661-8000
Fax.: (212) 661-8002

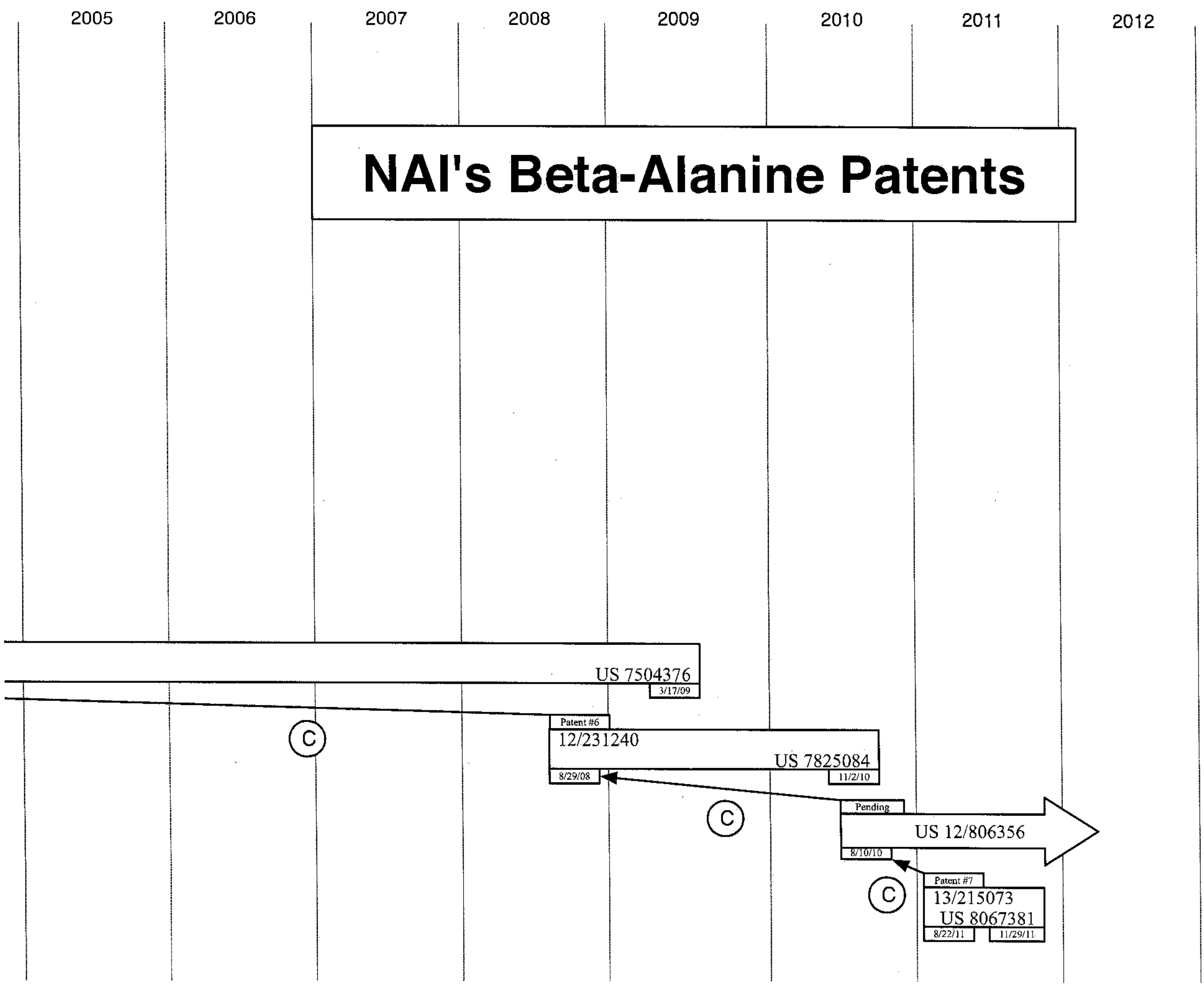
EXHIBIT A



Codes:

FP = Foreign Priority
D = Divisional
C = Continuation
P= Priority Claimed

Filing Dates - below boxes on left
Issue/Termination Dates - below boxes on right


2005
2006
2007
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2009
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2012
NAI's Beta-Alanine Patents
US 7504376
3/17/09
Patent #6
12/231240
US 7825084
8/29/08
11/2/10
C
C
Pending
US 12/806356
8/10/10
C
Patent #7
13/215073
US 8067381
8/22/11
11/29/11

EXHIBIT B

Attorney's Docket No.: 08457-002005

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant :	Harris, et al.	Art Unit :	1614
Serial No.:	10/717,217	Examiner :	Unknown
Filed	: November 18, 2003		
Title	: METHODS AND COMPOSITIONS FOR INCREASING THE ANAEROBIC WORKING CAPACITY IN TISSUES		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

TRANSMITTAL LETTER

Correspondence relating to this application is enclosed. The required fees are computed below. Please apply any charges not covered, or any credits, to Deposit Account No. 06-1050.

Total Claims	41	-	41	=	0	$0
Independent	8	-	8	=	0	$0
First Presentation of Multiple Dependent Claims						$0
TOTAL FEE DUE						$0

Respectfully submitted,

Date: 3/22/04

Joseph R. Baker, Jr.
Reg. No. 40,900

Fish & Richardson P.C.
PTO Customer No. 20985
12390 El Camino Real
San Diego, California 92130
Telephone: (858) 678-5070
Facsimile: (858) 678-5099
10378756.doc

CERTIFICATE OF MAILING BY FIRST CLASS MAIL

I hereby certify under 37 CFR §1.8(a) that this correspondence is being deposited with the United States Postal Service as first class mail with sufficient postage on the date indicated below and is addressed to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

March 22, 2004
Date of Deposit

Signature

Teri Barnett
Typed or Printed Name of Person Signing Certificate

Attorney's Docket No.: 08457-002005/

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Harris, et al.
Serial No.: 10/717,217
Filed : November 18, 2003
Title : METHODS AND COMPOSITIONS FOR INCREASING THE ANAEROBIC WORKING CAPACITY IN TISSUES

Art Unit: 1614
Examiner:

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

PRELIMINARY AMENDMENT

Prior to examination, please amend the application as follows:

Amendments to the Specification begin on page 2 of this paper.

Remarks begin on page 3 of this paper.

CERTIFICATE OF MAILING BY FIRST CLASS MAIL

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail with sufficient postage on the date indicated below and is addressed to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

March 22, 2004
Date of Deposit

Teri Barnett
Signature

Teri Barnett
Typed or Printed Name of Person Signing Certificate

Amendments to the Specification:

Please replace the paragraph beginning at page 1, line 4 with the following new paragraph:

-- CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of priority under 35 USC 119(e) of U.S. provisional application serial no. 60/462,238, filed April 10, 2003. This application also claims priority to U.S. application serial no. 10/209,169, filed July 30, 2002, which is a continuation of U.S. application serial no. 09/757,782, filed January 9, 2001, now U.S. Patent No. 6,426,361, which is a continuation of U.S. application serial no. 09/318,530, filed May 25, 1999, now U.S. Patent No. 6,172,098, which is a divisional of U.S. application serial no. 08/909,513, filed August 12, 1997, now U.S. Patent No. 5,965,596, which claims the benefit of foreign priority under 35 USC 119 to United Kingdom application nos. 9621914.2, filed October 21, 1996, and 9616910.7, filed August 12, 1996. The disclosure of the prior applications are considered part of (and is incorporated by reference in) the disclosure of this application.--

REMARKS

Applicant asks that all claims be examined. Please apply any other charges or credits to Deposit Account No. 06-1050.

Respectfully submitted,

Date: 3/22/04

Joseph R. Baker
Reg. No. 40,900

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San Diego, CA 92130-2081
Telephone: (858) 678-5070
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10378007.doc

EXHIBIT C

04-04-0403 RCE

BELL BOYD
BELL BOYD & LLOYD LLP

OIPE IAP2A SEP 0 2 2008 PATENT & TRADEMARK OFFICE

Substitute Form PTO/SB/30 (5-03)

Request For Continued Examination (RCE) Transmittal

Address to:
Mail Stop RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Application Number	10/717,217
Filing Date	November 18, 2003
First Named Inventor	Roger HARRIS
Group Art Unit	1614
Examiner Name	Raymond J. Henley III.
Customer Number	77202
Attorney Docket Number	1000141-00155 / 1412E

This is a Request for Continued Examination (RCE) under 37 C.F.R. §1.114 of the above-identified application.

1. Submission required under 37 C.F.R. §1.114
   - a. ☐ Previously submitted
     - i. ☐ Consider the amendment/reply under 37 C.F.R. 1.116 previously filed filed on ______
     - ii. ☐ Consider the arguments in the Appeal Brief or Reply Brief previously filed on ______
     - iii. ☐ Other ______
   - b. ☒ Enclosed
     - i. ☒ Amendment/Reply
     - ii. ☐ Affidavit(s)/Declaration(s)
     - iii. ☒ Information Disclosure Statement (IDS)
     - iv. ☒ Other: a Return Postcard
2. Miscellaneous
   - a. ☐ Suspension of action on the above-identified application is requested under 37 C.F.R. §1.103(c) for a period of ______ months. (Period of suspension shall not exceed 3 months; Fee under 37 C.F.R. §1.17(i) required)
   - b. ☐ Other ______
3. Fee — The RCE fee under 37 C.F.R. §1.17(e) is required by 37 C.F.R. §1.114 when the RCE is filed.
   - a. ☒ The Director is hereby authorized to charge the fees set forth below, and any other fees that may be due in connection with this and the attached papers or with this application during its entire pendency, or to credit any overpayments, to Deposit Account No. 02-1818. A duplicate of this sheet is enclosed.
     - i. ☒ RCE fee required under 37 CFR 1.17(e) - $405
     - ii. ☐ Extension of time fee (37 CFR 1.136 and 1.17) -
     - iii. ☒ Other Any deficiencies
   - b. ☐ Check in the amount of $____ enclosed
   - c. ☐ Payment by credit card (Form PTO-2038 enclosed)

SIGNATURE OF APPLICANT, ATTORNEY OR AGENT REQUIRED

Name (Print/Type)	Stephanie Seidman	*Registration No. (Attorney/Agent)*	33,779
Signature		*Date*	September 2, 2008

CERTIFICATE OF MAILING BY "EXPRESS MAIL"

I hereby certify that this paper and any attached papers are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" under "Express Mail" Mailing Label Number EM 247736986 US in an envelope addressed to: Mail Stop RCE, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on the date shown below.

Name (Print/Type)	Steven Dennis		
Signature		*Date*	September 2, 2008

09/04/2008 LTRUONG 00000034 021818 10717217

01 FC:2801 405.00 DA

BELL BOYD
BELL BOYD & LLOYD LLP

Substitute Form PTO/SB/30 (5-03)

Request For Continued Examination (RCE) Transmittal

Address to:
Mail Stop RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

OIPE SEP 0 2 2008 PATENT & TRADEMARK OFFICE

Application Number	10/717,217
Filing Date	November 18, 2003
First Named Inventor	Roger HARRIS
Group Art Unit	1614
Examiner Name	Raymond J. Henley III.
Customer Number	77202
Attorney Docket Number	1000141-00155 / 1412E

This is a Request for Continued Examination (RCE) under 37 C.F.R. §1.114 of the above-identified application.

1. Submission required under 37 C.F.R. §1.114
   a. ☐ Previously submitted
      i. ☐ Consider the amendment/reply under 37 C.F.R. 1.116 previously filed filed on ______
      ii. ☐ Consider the arguments in the Appeal Brief or Reply Brief previously filed on ______
      iii. ☐ Other ______
   b. ☒ Enclosed
      i. ☒ Amendment/Reply
      ii. ☐ Affidavit(s)/Declaration(s)
      iii. ☒ Information Disclosure Statement (IDS)
      iv. ☒ Other: a Return Postcard

2. Miscellaneous
   a. ☐ Suspension of action on the above-identified application is requested under 37 C.F.R. §1.103(c) for a period of ______ months. (Period of suspension shall not exceed 3 months; Fee under 37 C.F.R. §1.17(i) required)
   b. ☐ Other ______

3. Fee The RCE fee under 37 C.F.R. §1.17(e) is required by 37 C.F.R. §1.114 when the RCE is filed.
   a. ☒ The Director is hereby authorized to charge the fees set forth below, and any other fees that may be due in connection with this and the attached papers or with this application during its entire pendency, or to credit any overpayments, to Deposit Account No. 02-1818. A duplicate of this sheet is enclosed.
      i. ☒ RCE fee required under 37 CFR 1.17(e) - $405
      ii. ☐ Extension of time fee (37 CFR 1.136 and 1.17) -
      iii. ☒ Other Any deficiencies
   b. ☐ Check in the amount of $____ enclosed
   c. ☐ Payment by credit card (Form PTO-2038 enclosed)

SIGNATURE OF APPLICANT, ATTORNEY OR AGENT REQUIRED

Name (Print/Type)	Stephanie Seidman	*Registration No. (Attorney/Agent)*	33,779
Signature		*Date*	September 2, 2008

CERTIFICATE OF MAILING BY "EXPRESS MAIL"

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Name (Print/Type)	Steven Dennis		
Signature		*Date*	September 2, 2008

Attorney's Docket No.: 1000141-00155 / 1412E

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	: Harris *et al.*	Art Unit	: 1614
Serial No.	: 10/717,217	Examiner	: Raymond J. Henley III
Filed	: November 18, 2003	Confirm. No.:	6038

Title : **METHODS AND COMPOSITIONS FOR INCREASING THE ANAEROBIC WORKING CAPACITY IN TISSUES**

Mail Stop RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

PRELIMINARY AMENDMENT AND REQUEST FOR CONTINUED EXAMINATION (RCE)

Dear Sir:

This preliminary amendment is filed with a Request for Continued Examination (RCE) of the above-captioned application. Entry of the following amendments and consideration of the following remarks are respectfully requested.

Amendments to the specification begin on page 2 of this paper.

Amendments to the claims are reflected in the listing of the claims, which begin on page 3 of this paper.

Remarks/Arguments begin on page 11 of this paper.

A **Supplemental Information Disclosure Statement** accompanies this response.

CERTIFICATE OF MAILING BY "EXPRESS MAIL"
"Express Mail" Mailing Label Number EM 247736986 US
Date of Deposit: **September 2, 2008**
I hereby certify that this paper is being deposited with the United States Postal "Express Mail Post Office to Addressee" Service under 37 CFR §1.10 on the date indicated above and is addressed to: Mail Stop RCE, Commissioner for Patents, U.S. Patent and Trademark Office, P.O. Box 1450, Alexandria, VA, 22313-1450.

Steven Dennis

AMENDMENT TO THE SPECIFICATION:

Please amend the specification at page 1, under "Related Application," lines 4-26 as follows:

This application claims the benefit of priority under 35 U.S.C. Section 119(e) of U.S. Provisional Application No. 60/462,238, filed Apr. 10, 2003, which is incorporated by reference herein. ~~This application~~

The following applications also are incorporated by reference herein: ~~is also a continuation-in-part (CIP) of~~ U.S. application Ser. No. 10/209,169, filed Jul. 30, 2002; ~~,~~ ~~which is a continuation of~~ U.S. application Ser. No. 09/757,782, filed Jan. 9, 2001, now U.S. Pat. No. 6,426,361; ~~, which is a continuation of~~ U.S. application Ser. No. 09/318,530, filed May 25, 1999, now U.S. Pat. No. 6,172,098; ~~, which is a divisional of~~ U.S. application Ser. No. 08/909,513, filed Aug. 12, 1997, now U.S. Pat. No. 5,965,596; and ~~, which claims the benefit of foreign priority under 35 USC 119 to~~ United Kingdom application nos. 9621914.2, filed Oct. 21, 1996, and 9616910.7, filed Aug. 12, 1996. ~~The aforementioned applications are explicitly incorporated herein by reference in their entirety and for all purposes.~~

AMENDMENTS TO THE CLAIMS:

Claims 5, 7, 8 and 11-22 and 42-44 are pending. Please add new claims 42-44. This listing of claims replaces all prior versions and listings of claims in the application.

LISTING OF CLAIMS:

1. - 4. (Cancelled).

5. (Currently amended) A composition, comprising:

glycine; and

a) an amino acid selected from the group consisting of a beta-alanine, an ester of a beta-alanine, and an amide of a beta-alanine, or

b) a di-peptide selected from the group consisting of a beta-alanine di-peptide and a beta-alanylhistidine di-peptide.

6. (Cancelled).

7. (Currently amended) The composition of claim 5, wherein the beta-alanylhistidine dipeptide ~~comprises~~ is a carnosine, an anserine or a balenine.

8. (Previously presented) The composition of claim 5, further comprising at least creatine or a carbohydrate.

9 and 10. (Cancelled).

11. (Previously presented) The composition of claim 5, wherein the composition is a pharmaceutical composition.

12. (Previously presented) The composition of claim 5, wherein the composition is a dietary supplement or a sports drink.

13. (Previously presented) The composition of claim 12, wherein the dietary supplement or sports drink is a supplement for humans.

14. (Currently amended) A composition comprising at least 0.2, 0.3, 0.4, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 or 5 grams of a peptide or an ester comprising a beta-alanine per dosage.

15. (Currently amended) A composition comprising at least 0.2, 0.3, 0.4, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 grams of a peptide or an ester comprising a beta-alanine in an injectable form per dosage.

16. (Previously presented) The composition of claim 14 or claim 15, wherein the peptide comprises a beta-alanylhistidine dipeptide.

17. (Previously presented) The composition of claim 16, wherein the beta-alanylhistidine dipeptide comprises a carnosine, an anserine or a balenine.

18. (Currently amended) A composition for humans comprising at least 200, 250, 300, 450, 500, 550, 600, 650, 700, 750 or 800 mg of a beta-alanine per dosage.

19. (Previously presented) The composition of claim 18, wherein the composition is formulated in an ingestible or an injectable formulation.

20. (Previously presented) The composition of claim 19, wherein the ingestible formulation is a drink, a gel, a food or a tablet.

21. (Previously presented) The composition of claim 18, wherein the peptide comprises a beta-alanylhistidine dipeptide.

22. (Previously presented) The composition of claim 20, wherein the beta-alanylhistidine dipeptide comprises a carnosine, an anserine or a balenine.

23. - 41. (Cancelled).

42. (New) The composition of claim 5, wherein the composition is formulated for oral, enteral or parenteral administration.

43. (New) The composition of claim 5, wherein the composition is formulated for infusion through the skin of a subject.

44. (New) The composition of claim 43, wherein the composition is a topical cream or a patch.

REMARKS

The requisite fee for filing a Request for Continued Examination and any other fees that may be due in connection with the filing of this paper or with this application should be charged to Deposit Account No. 02-1818. If a Petition for Extension of Time is needed, this paper is to be considered such Petition. A supplemental Information Disclosure Statement is filed herewith.

Claims 5, 7, 8, 11-22 and 42-44 are pending. Claims 42-44 are added. Claims 5, 7, 8 and 11-22 previously were allowed. Claim 5 is amended to correct formatting. Claims 7, 14, 15 and 18 are amended for clarity. Basis for new claims 42-44 can be found throughout the specification (*e.g.*, see page 13, lines 9-11). No new matter is added.

PRIORITY CLAIM

The specification is amended to correct the priority claim. A Request for Corrected Filing Receipt will be filed under separate cover.

* * *

In view of the amendment and remarks herein, allowance of the application respectfully is requested.

Respectfully submitted,

Stephanie Seidman
Reg. No. 33,779

Attorney Docket No. 100141-00155 / 1412E
Address all correspondence to:
77202
Stephanie Seidman
Bell, Boyd & Lloyd LLP
3580 Carmel Mountain Road, Suite 200
San Diego, California 92130
Telephone: (858) 509-7410
Facsimile: (858) 509-7460
email: sseidman@bellboyd.com

EXHIBIT D

Attorney's Docket No.: 1000141-00155 / 1412E

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Harris *et al.*
Serial No. : 10/717,217
Filed : November 18, 2003
Cust. No. : 77202
Title : **METHODS AND COMPOSITIONS FOR INCREASING THE ANAEROBIC WORKING CAPACITY IN TISSUES**

Art Unit : 1614
Examiner : Henley III, Raymond J.
Conf. No. : 6038

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

REQUEST FOR CORRECTED UPDATED FILING RECEIPT

Dear Sir:

Attached is a copy of the updated filing receipt received from the PTO in the above-captioned application for which issuance of a corrected updated filing receipt is respectfully requested.

Please change the **ATTORNEY DOCKET** information to read as follows:

1000141-00155 / 1412E

Please change the **CORRESPONDENCE ADDRESS** information to read as follows:

77202
BELL, BOYD & LLOYD LLP
3580 CARMEL MOUNTAIN ROAD
SUITE 200
SAN DIEGO, CA 92130

Please change the **DOMESTIC PRIORITY DATA AS CLAIMED BY APPLICANT** information to read as follows:

This application claims benefit of 60/462,238 04/10/02003

To evidence this, attached is a copy of the *Preliminary Amendment* amending the priority claim.

Please delete the **FOREIGN APPLICATIONS** information. To evidence this, attached is a copy of the *Preliminary Amendment* amending the priority claim.

CERTIFICATE OF MAILING BY FIRST CLASS MAIL

I hereby certify under 37 CFR § 1.8(a) that this correspondence is being deposited with the United States Postal Service as first class mail with sufficient postage on the date indicated below and is addressed to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

October 1, 2008	John L. Macalagay	John L. Macalagay
Date of Deposit	Signature	Typed Name of Person Signing Certificate

1003722/D/2

Applicant respectfully requests corrections of these errors and provision of a new Official Filing Receipt.

Respectfully submitted,

Stephanie Seidman
Reg. No. 33,779

Attorney's Docket No.: 1000141-00155 / 1412E
Address all correspondence to: 77202
STEPHANIE SEIDMAN
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3580 CARMEL MOUNTAIN ROAD, SUITE 200
SAN DIEGO, CALIFORNIA 92130
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Facsimile: (858) 509-7460
email: sseidman@bellboyd.com

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPL NO.	FILING OR 371 (c) DATE	ART UNIT	FIL FEE REC'D	ATTY.DOCKET NO	DRAWINGS	TOT CLMS	IND CLMS
10/717,217	11/18/2003	1614	1017	~~08457-002005~~ 1000141-00155 / 1412E	19	41	8

CONFIRMATION NO. 6038

~~20985~~
FISH & ~~RICHARDSON, PC~~
12390 ~~EL CAMINO REAL~~
~~SAN DIEGO, CA 92130-2081~~

77202
BELL, BOYD & LLOYD LLP
3580 CARMEL MOUNTAIN ROAD
SUITE 200
SAN DIEGO, CA 92130

UPDATED FILING RECEIPT

OC000000013473923

Date Mailed: 08/09/2004

Receipt is acknowledged of this regular Patent Application. It will be considered in its order and you will be notified as to the results of the examination. Be sure to provide the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION when inquiring about this application. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please write to the Office of Initial Patent Examination's Filing Receipt Corrections, facsimile number 703-746-9195. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections (if appropriate).**

Applicant(s)

Roger Harris, New Market, UNITED KINGDOM;
Mark Dunnett, Tuddenham, UNITED KINGDOM;

Assignment For Published Patent Application

NATURAL ALTERNATIVES INTERNATIONAL, San Marcos, CA;

Domestic Priority data as claimed by applicant

This appln claims benefit of 60/462,238 04/10/2003
~~and is a CIP of 10/209,169 07/30/2002 PAT 6,680,294~~
~~which is a CON of 09/757,782 01/09/2001 PAT 6,426,361~~
~~which is a CON of 09/318,530 05/25/1999 PAT 6,172,098~~
~~which is a DIV of 08/909,513 08/12/1997 PAT 5,965,596~~

Foreign Applications

~~UNITED KINGDOM 9621914.2 10/21/1996~~
~~UNITED KINGDOM 9616910.7 08/12/1996~~

If Required, Foreign Filing License Granted: 03/03/2004

Projected Publication Date: 11/18/2004

Non-Publication Request: No

Early Publication Request: No

** SMALL ENTITY **

Title

Methods and compositions for increasing the anaerobic working capacity in tissues

Preliminary Class

514

LICENSE FOR FOREIGN FILING UNDER
Title 35, United States Code, Section 184
Title 37, Code of Federal Regulations, 5.11 & 5.15

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OIPE JCS55 MAR 2 4 2004 PATENT & TRADEMARK OFFICE

Attorney's Docket No.: 08457-002005

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant :	Harris, et al.	Art Unit :	1614
Serial No.:	10/717,217	Examiner :	Unknown
Filed	: November 18, 2003		
Title	: METHODS AND COMPOSITIONS FOR INCREASING THE ANAEROBIC WORKING CAPACITY IN TISSUES		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

TRANSMITTAL LETTER

Correspondence relating to this application is enclosed. The required fees are computed below. Please apply any charges not covered, or any credits, to Deposit Account No. 06-1050.

Total Claims	41	-	41	=	0		$0
Independent	8	-	8	=	0		$0
First Presentation of Multiple Dependent Claims							$0
TOTAL FEE DUE							$0

Respectfully submitted,

Date: 3/22/04

Joseph R. Baker, Jr.
Reg. No. 40,900

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I hereby certify under 37 CFR §1.8(a) that this correspondence is being deposited with the United States Postal Service as first class mail with sufficient postage on the date indicated below and is addressed to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

March 22, 2004
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Signature

Teri Barnett
Typed or Printed Name of Person Signing Certificate

Attorney's Docket No.: 08457-002005/

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Harris, et al. Art Unit: 1614
Serial No.: 10/717,217 Examiner:
Filed : November 18, 2003
Title : METHODS AND COMPOSITIONS FOR INCREASING THE ANAEROBIC WORKING CAPACITY IN TISSUES

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

PRELIMINARY AMENDMENT

Prior to examination, please amend the application as follows:

Amendments to the Specification begin on page 2 of this paper.

Remarks begin on page 3 of this paper.

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March 22, 2004
Date of Deposit

Teri Barnett
Signature

Teri Barnett
Typed or Printed Name of Person Signing Certificate

Amendments to the Specification:

Please replace the paragraph beginning at page 1, line 4 with the following new paragraph:

-- CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of priority under 35 USC 119(e) of U.S. provisional application serial no. 60/462,238, filed April 10, 2003. This application also claims priority to U.S. application serial no. 10/209,169, filed July 30, 2002, which is a continuation of U.S. application serial no. 09/757,782, filed January 9, 2001, now U.S. Patent No. 6,426,361, which is a continuation of U.S. application serial no. 09/318,530, filed May 25, 1999, now U.S. Patent No. 6,172,098, which is a divisional of U.S. application serial no. 08/909,513, filed August 12, 1997, now U.S. Patent No. 5,965,596, which claims the benefit of foreign priority under 35 USC 119 to United Kingdom application nos. 9621914.2, filed October 21, 1996, and 9616910.7, filed August 12, 1996. The disclosure of the prior applications are considered part of (and is incorporated by reference in) the disclosure of this application.--

Attorney's Docket No.:08457-002005

REMARKS

Applicant asks that all claims be examined. Please apply any other charges or credits to Deposit Account No. 06-1050.

Respectfully submitted,

Date: 3/22/04

Joseph R. Baker
Reg. No. 40,900

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EXHIBIT E

UNITED STATES PATENT AND TRADEMARK OFFICE

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United States Patent and Trademark Office
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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
10/717,217	11/18/2003	1614	1317	1000141-00155 / 1412E	41	8

CONFIRMATION NO. 6038

CORRECTED FILING RECEIPT

77202
Bell, Boyd & Lloyd LLP
3580 Carmel Mountain Road
Suite 200
San Diego, CA 92130

OC000000032945814

Date Mailed: 02/05/2009

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Applicant(s)
Roger Harris, New Market, UNITED KINGDOM;
Mark Dunnett, Tuddenham, UNITED KINGDOM;

Assignment For Published Patent Application
NATURAL ALTERNATIVES INTERNATIONAL, San Marcos, CA

Power of Attorney: The patent practitioners associated with Customer Number 77202

Domestic Priority data as claimed by applicant
This appln claims benefit of 60/462,238 04/10/2003

Foreign Applications

If Required, Foreign Filing License Granted: 03/03/2004

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 10/717,217**

Projected Publication Date: Not Applicable

Non-Publication Request: No

Early Publication Request: No
** SMALL ENTITY **

Title

METHODS AND COMPOSITIONS FOR INCREASING THE ANAEROBIC WORKING CAPACITY IN TISSUES

Preliminary Class

514

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Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4158).

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EXHIBIT F

US007504376B2

(12) **United States Patent**
Harris et al.

(10) **Patent No.: US 7,504,376 B2**
(45) **Date of Patent: Mar. 17, 2009**

(54) **METHODS AND COMPOSITIONS FOR INCREASING THE ANAEROBIC WORKING CAPACITY IN TISSUES**

(75) Inventors: **Roger Harris**, New Market (GB); **Mark Dunnett**, Tuddenham (GB)

(73) Assignee: **Natural Alternatives International**, San Marcos, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 493 days.

(21) Appl. No.: **10/717,217**

(22) Filed: **Nov. 18, 2003**

(65) **Prior Publication Data**

US 2004/0229773 A1 Nov. 18, 2004

Related U.S. Application Data

(60) Provisional application No. 60/462,238, filed on Apr. 10, 2003.

(30) **Foreign Application Priority Data**

Aug. 12, 1996 (GB) 9616910.7
Oct. 21, 1996 (GB) 9621914.2

(51) **Int. Cl.**
A61K 38/00 (2006.01)
A61K 31/415 (2006.01)

(52) **U.S. Cl.** **514/3**; 514/400

(58) **Field of Classification Search** .... None
See application file for complete search history.

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6,680,294	B2	1/2004	Harris et al.	

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(Continued)

Primary Examiner—Raymond J Henley, III
(74) *Attorney, Agent, or Firm*—Bell, Boyd & Lloyd LLP; Stephanie Seidman; Frank J. Miskiel

(57) **ABSTRACT**

The invention provides compositions comprising beta-alanylhistidine peptides and beta-alanines, and methods for administering these peptides and amino acids. In one aspect, the compositions and methods cause an increase in the blood plasma concentrations of beta-alanine and/or creatine.

18 Claims, 19 Drawing Sheets

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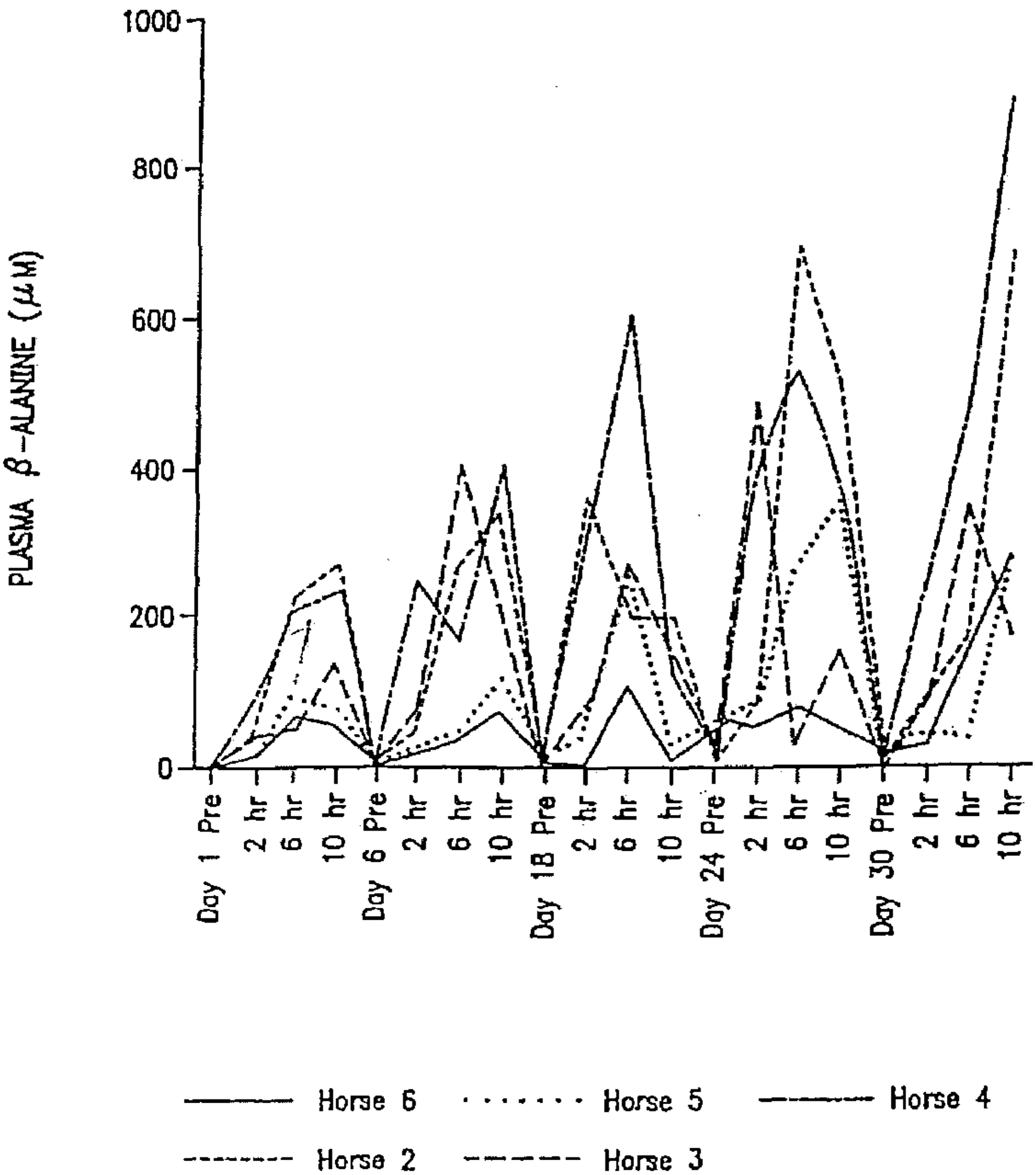
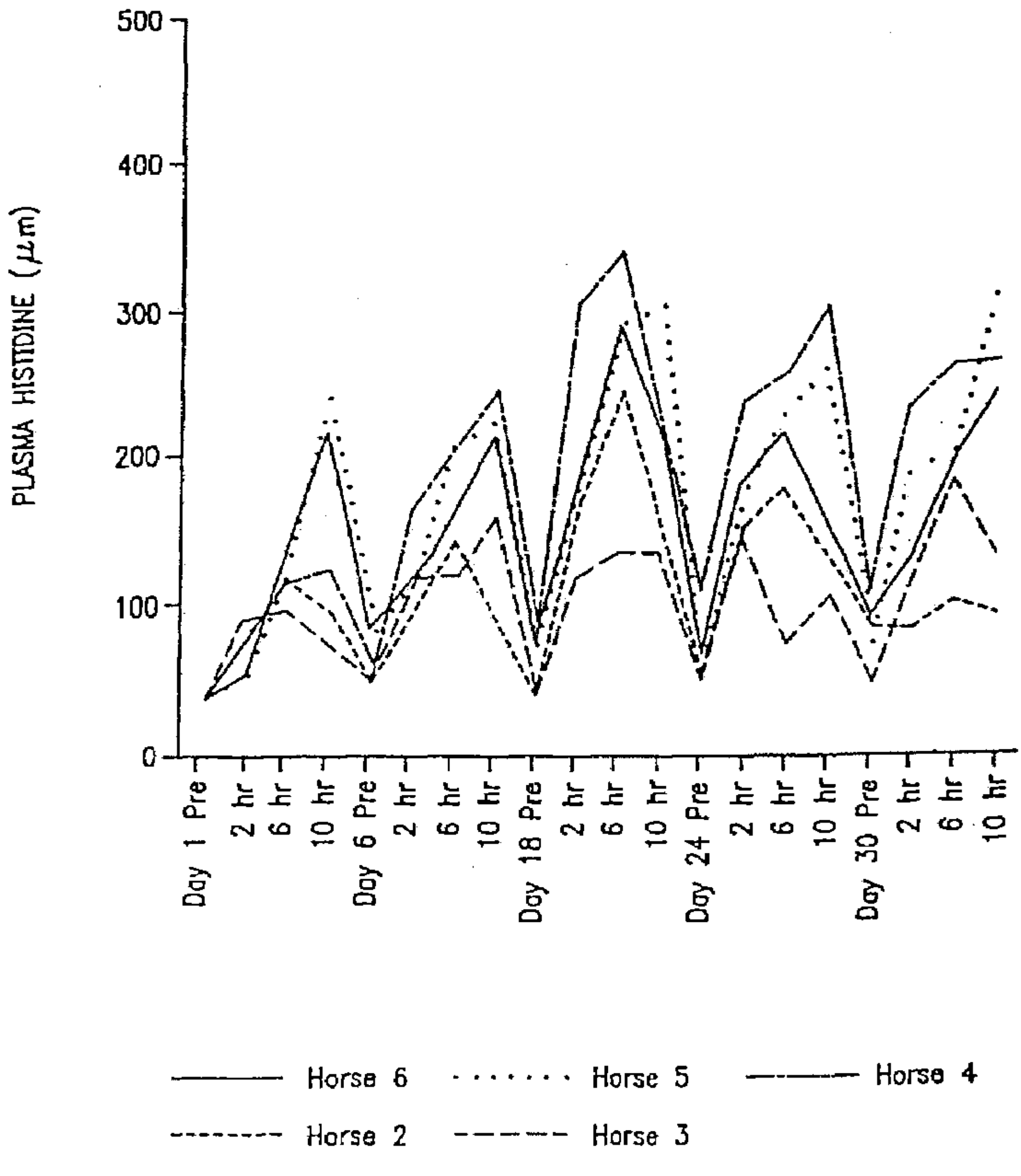

PLASMA β-ALANINE (μM)
1000
800
600
400
200
0
Day 1 Pre
2 hr
6 hr
10 hr
Day 6 Pre
2 hr
6 hr
10 hr
Day 18 Pre
2 hr
6 hr
10 hr
Day 24 Pre
2 hr
6 hr
10 hr
Day 30 Pre
2 hr
6 hr
10 hr
Horse 6
Horse 5
Horse 4
Horse 2
Horse 3

FIG. 1



500
400
300
200
100
0
PLASMA HISTIDINE (μm)
Day 1 Pre
2 hr
6 hr
10 hr
Day 6 Pre
2 hr
6 hr
10 hr
Day 18 Pre
2 hr
6 hr
10 hr
Day 24 Pre
2 hr
6 hr
10 hr
Day 30 Pre
2 hr
6 hr
10 hr
Horse 6
Horse 5
Horse 4
Horse 2
Horse 3

FIG. 2

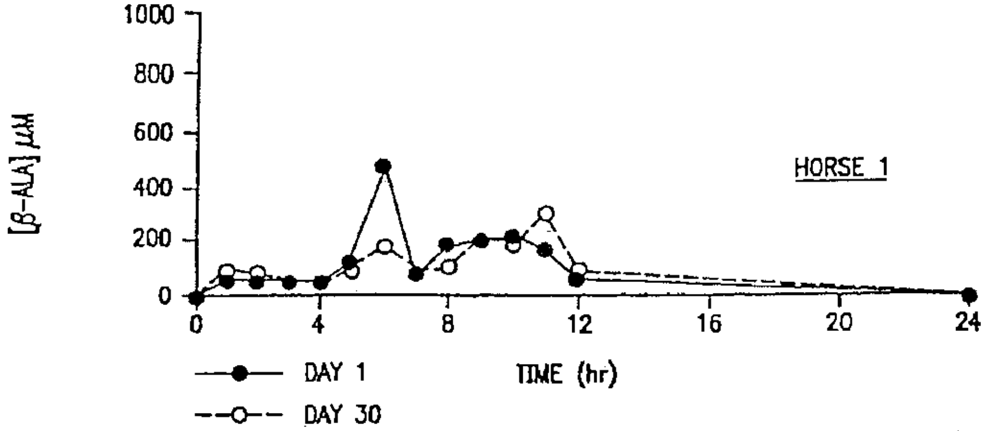


FIG. 3A

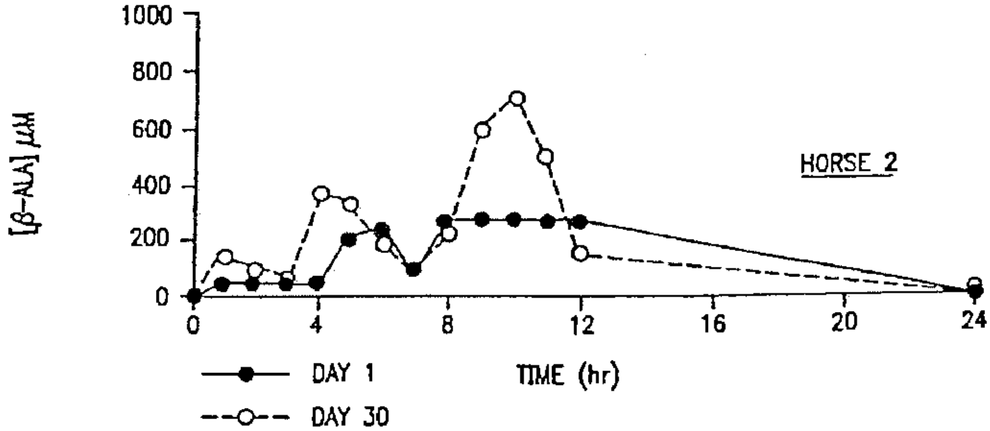


FIG. 3B

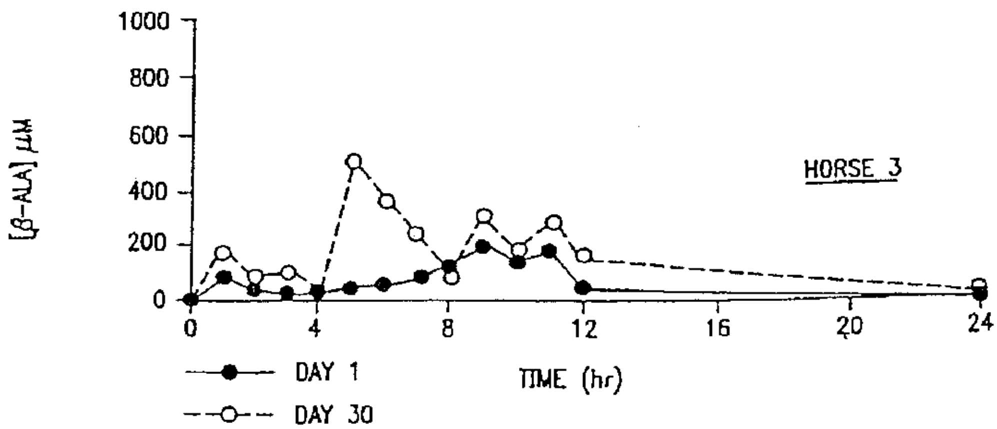


FIG. 3C

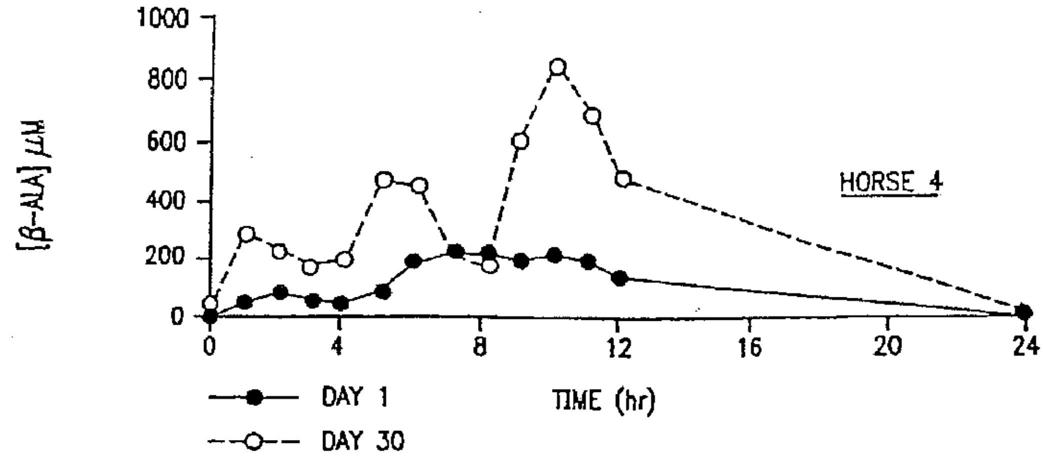


FIG. 3D

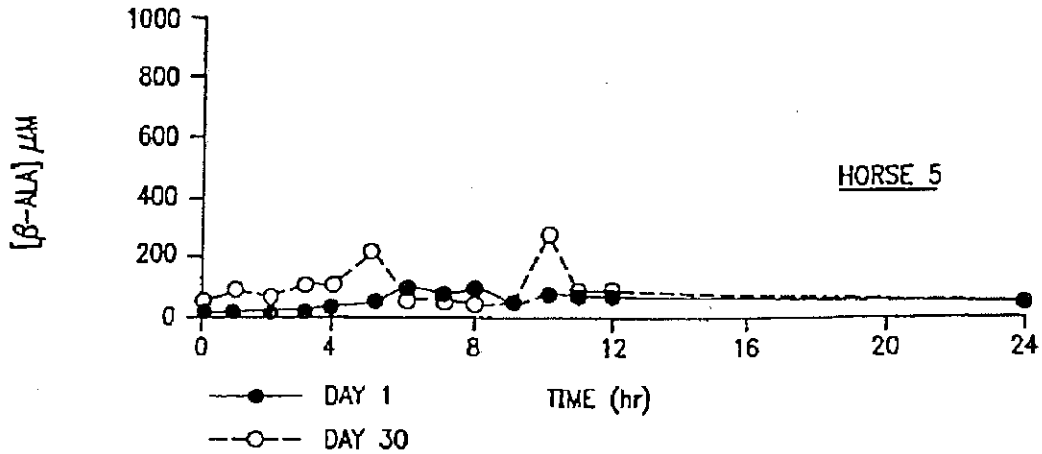


FIG. 3E

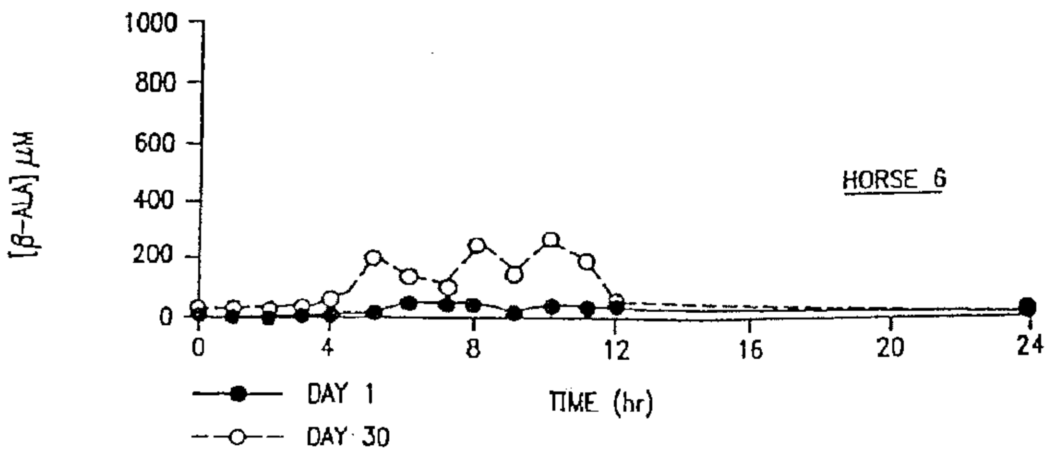


FIG. 3F

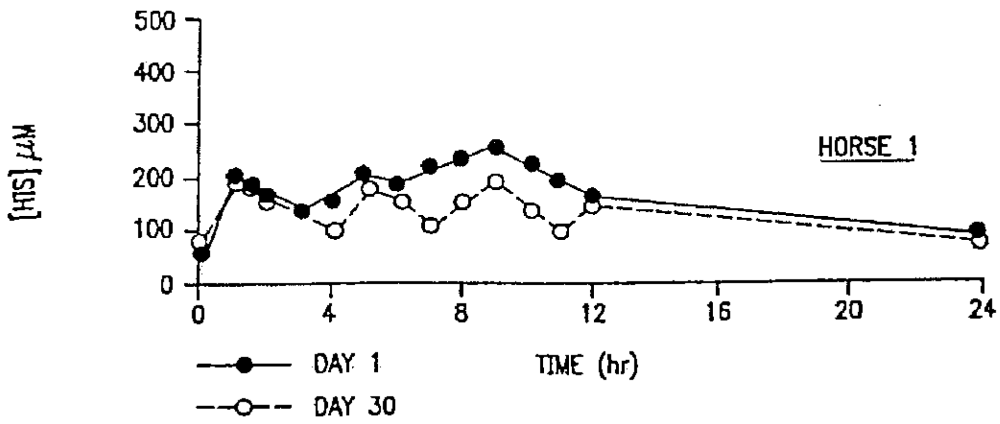


FIG. 4A

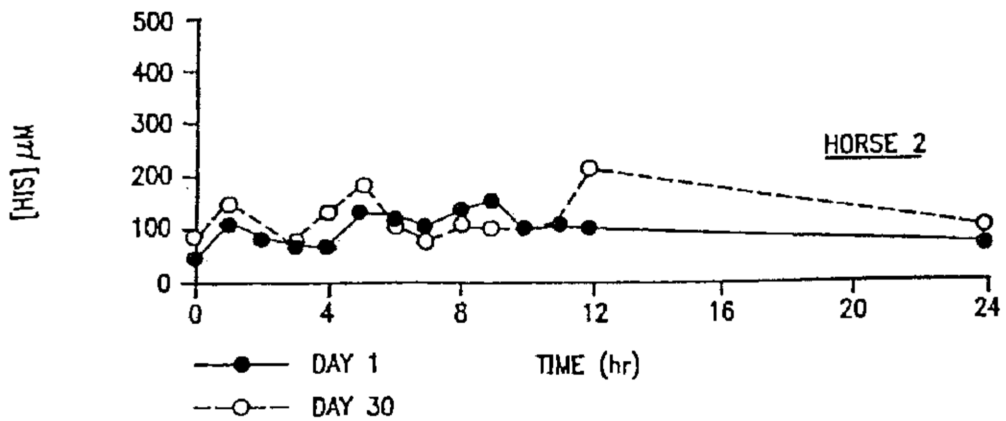


FIG. 4B

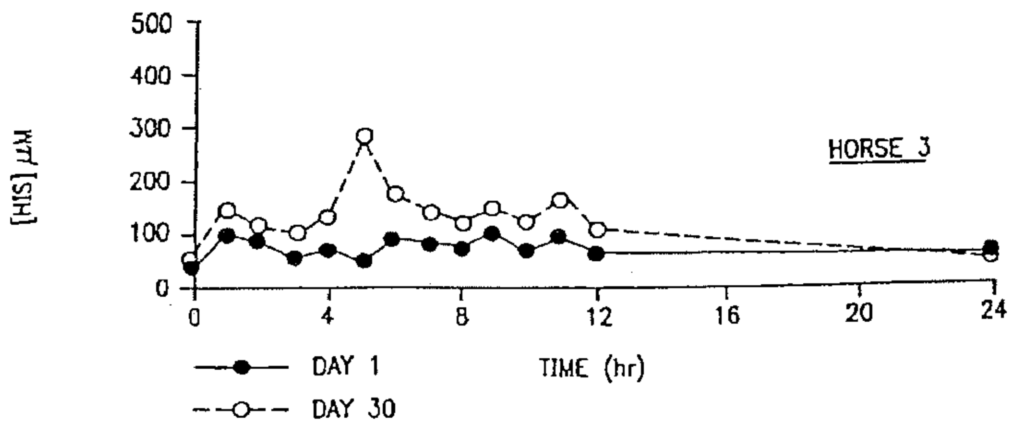


FIG. 4C

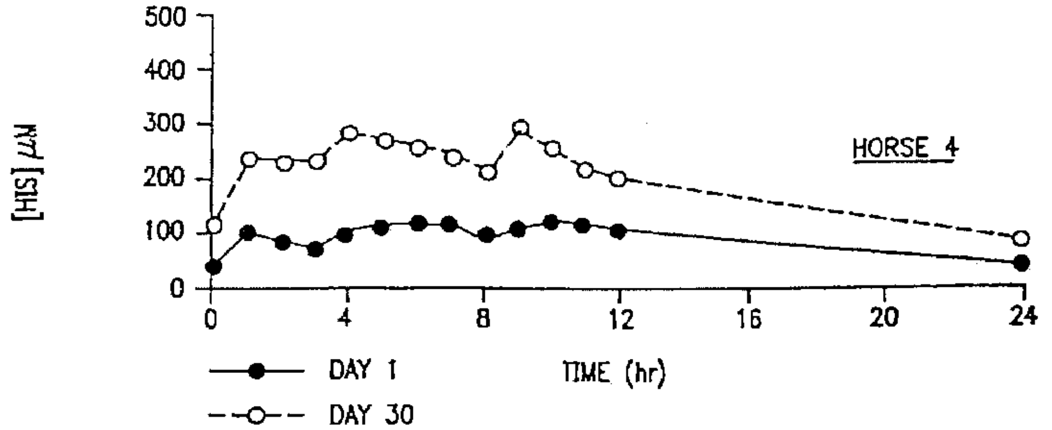


FIG. 4D

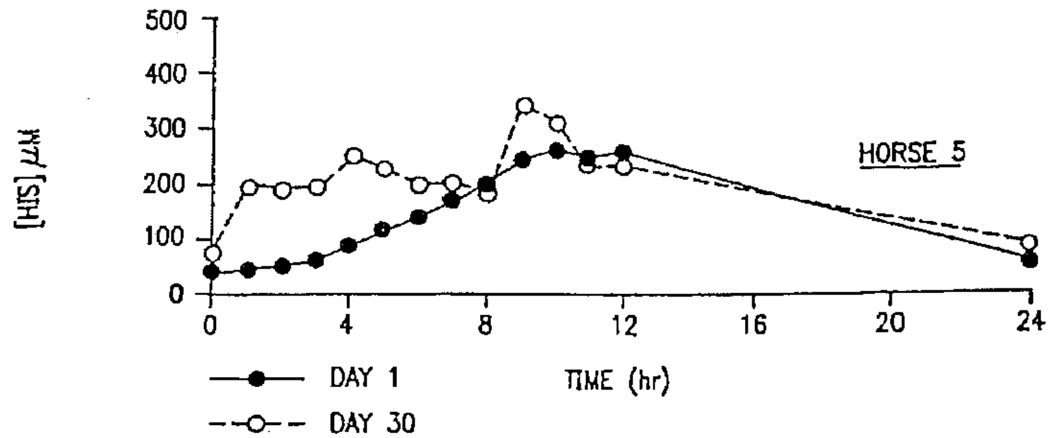


FIG. 4E

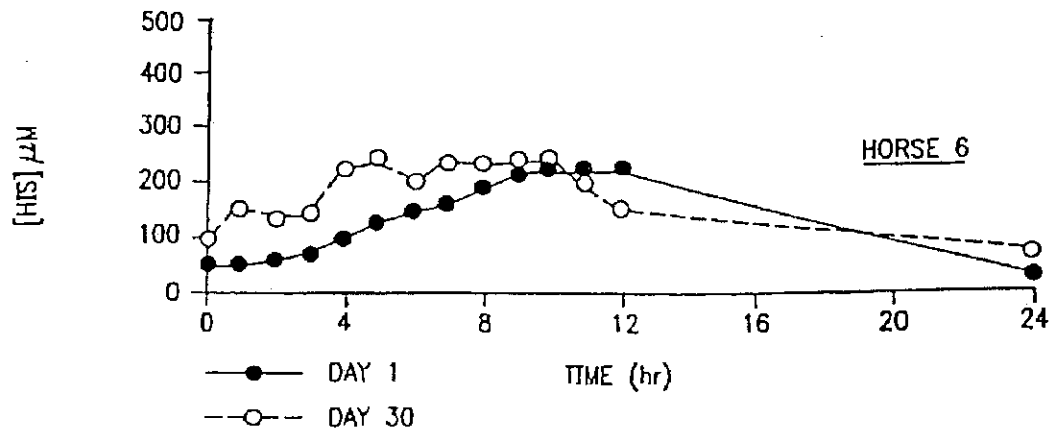
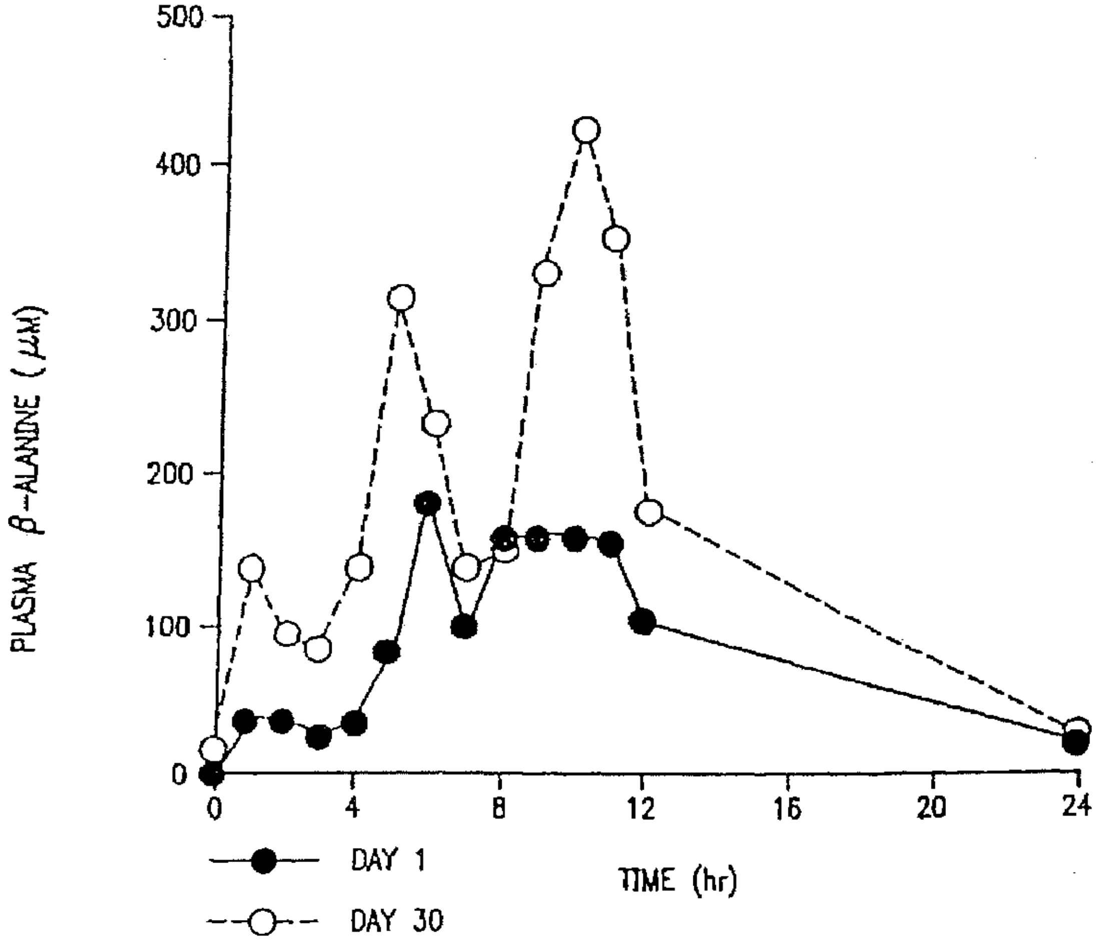


FIG. 4F



500
400
300
200
100
0
PLASMA β-ALANINE (μM)
0
4
8
12
16
20
24
TIME (hr)
DAY 1
DAY 30

FIG. 5

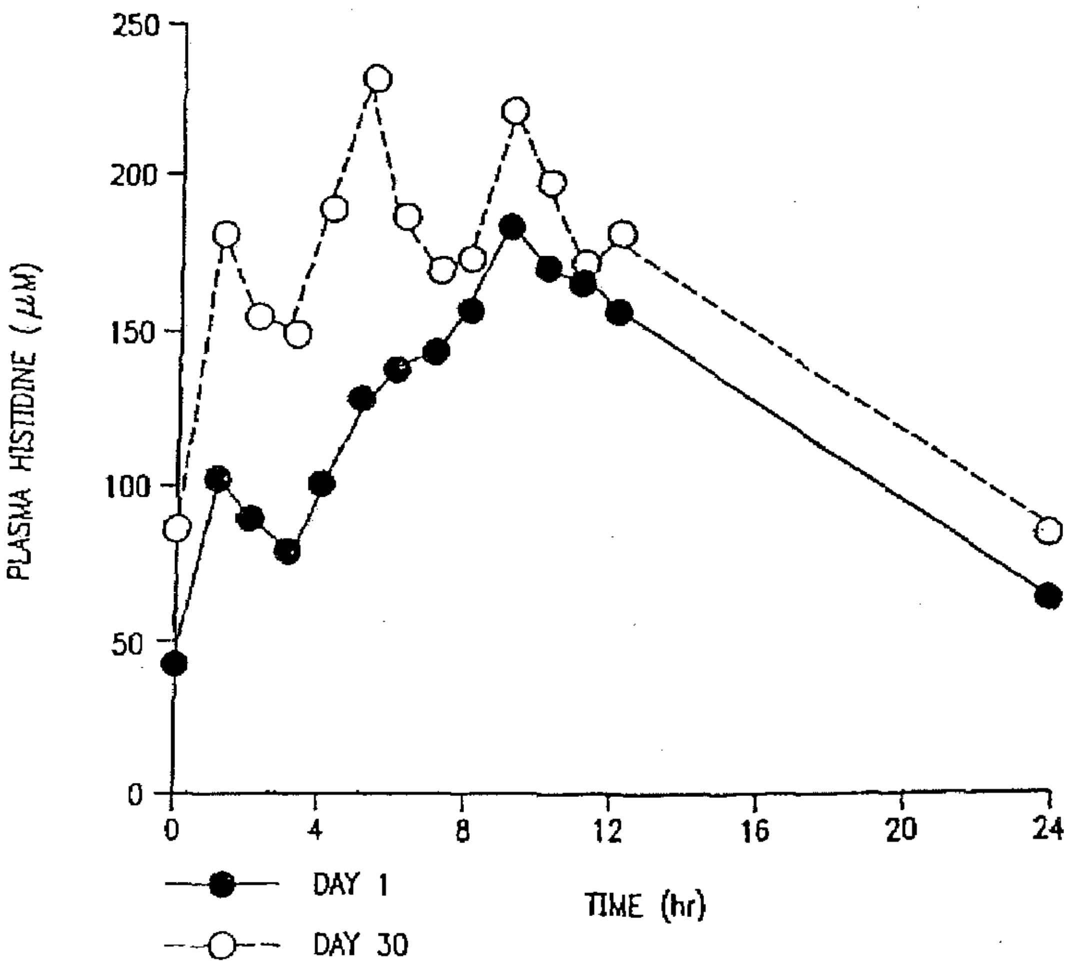
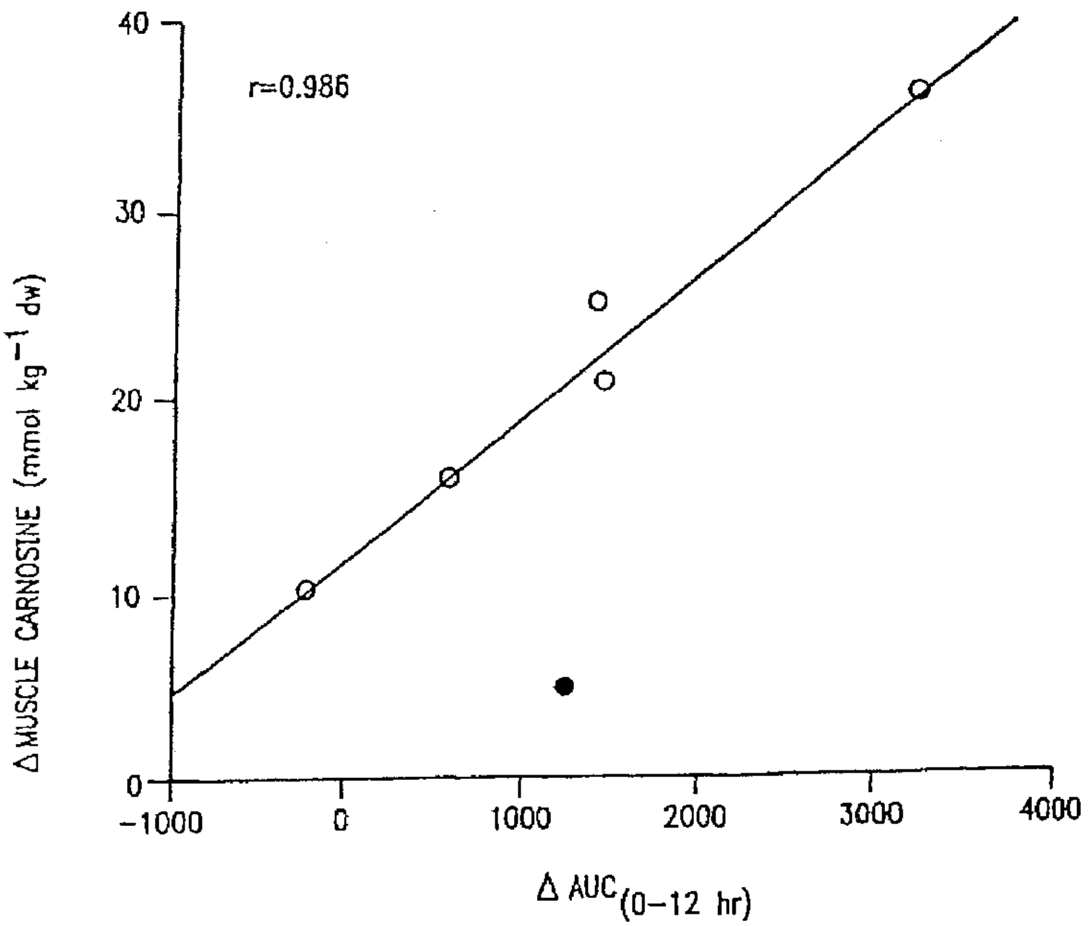

250
200
150
100
50
0
PLASMA HISTIDINE (μM)
0
4
8
12
16
20
24
TIME (hr)
DAY 1
DAY 30

FIG. 6



r=0.986
Δ MUSCLE CARNOSINE (mmol kg^{-1} dw)
40
30
20
10
0
−1000
0
1000
2000
3000
4000
Δ AUC$_{(0-12\ hr)}$

FIG. 7

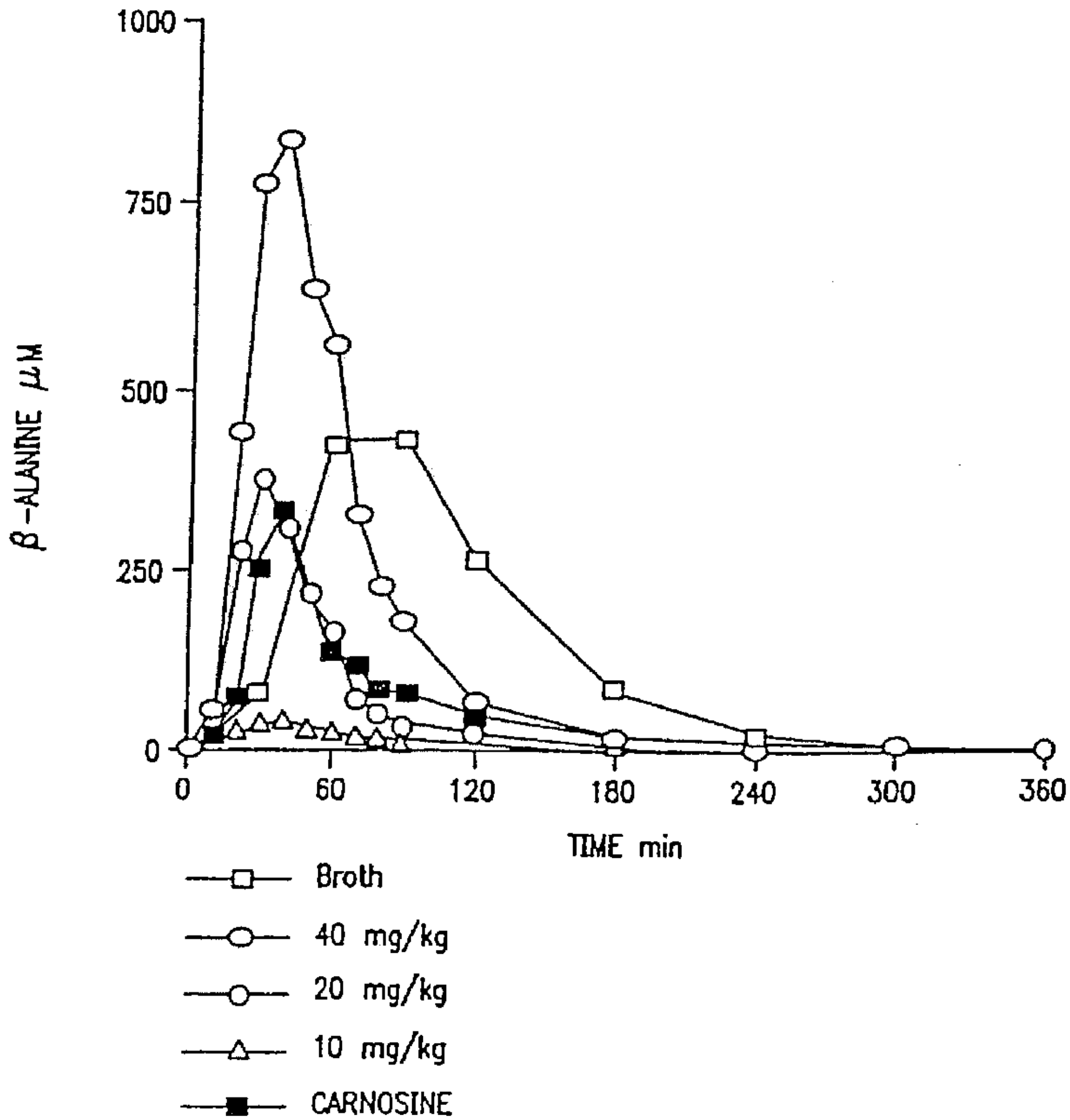

1000
750
500
250
0
β-ALANINE μM
0
60
120
180
240
300
360
TIME min
Broth
40 mg/kg
20 mg/kg
10 mg/kg
CARNOSINE

FIG. 8

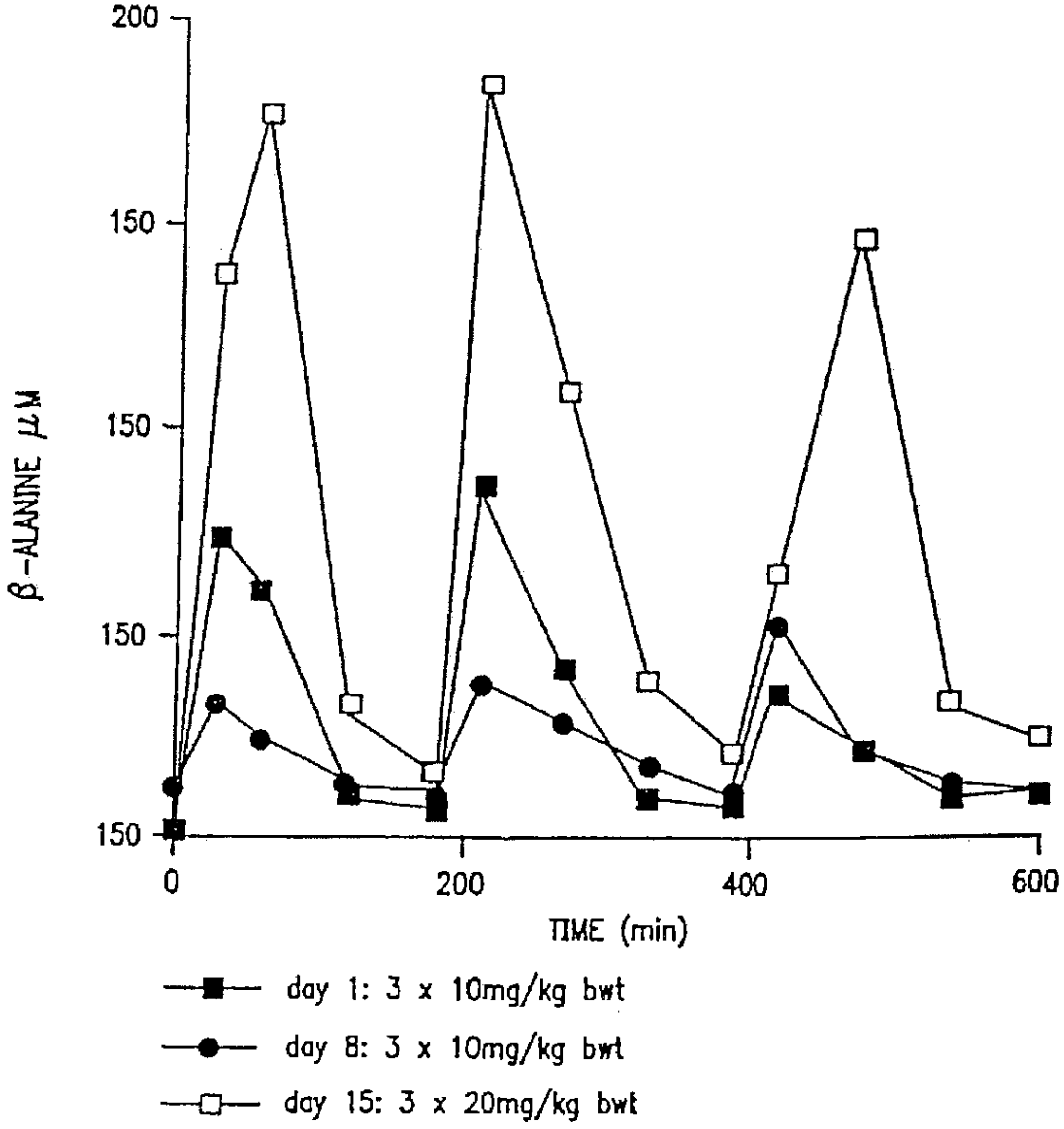

200
150
150
150
150
β-ALANINE μM
0
200
400
600
TIME (min)
day 1: 3 x 10mg/kg bwt
day 8: 3 x 10mg/kg bwt
day 15: 3 x 20mg/kg bwt

FIG. 9

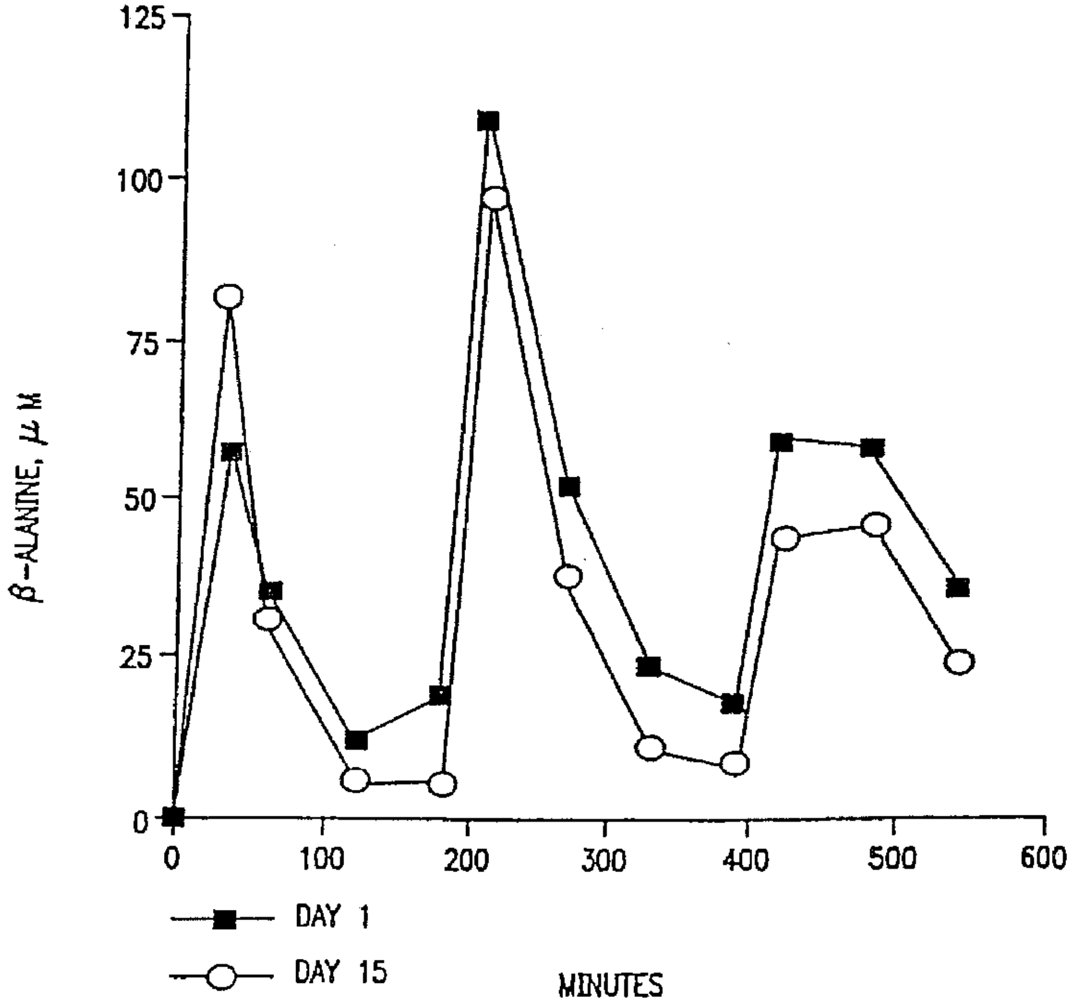

125
100
75
50
25
0
β-ALANINE, μM
0
100
200
300
400
500
600
DAY 1
DAY 15
MINUTES

FIG. 10

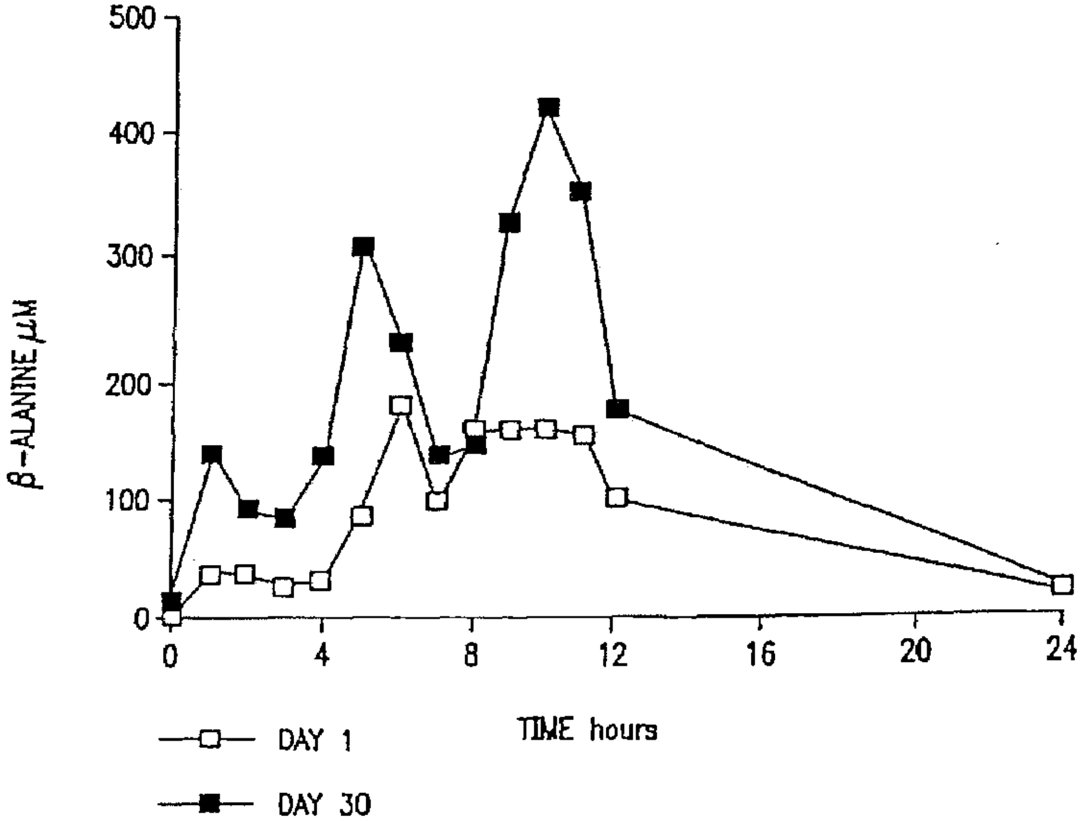

500
400
300
200
100
0
β-ALANINE μM
0
4
8
12
16
20
24
TIME hours
DAY 1
DAY 30

FIG. 11

Figure 12

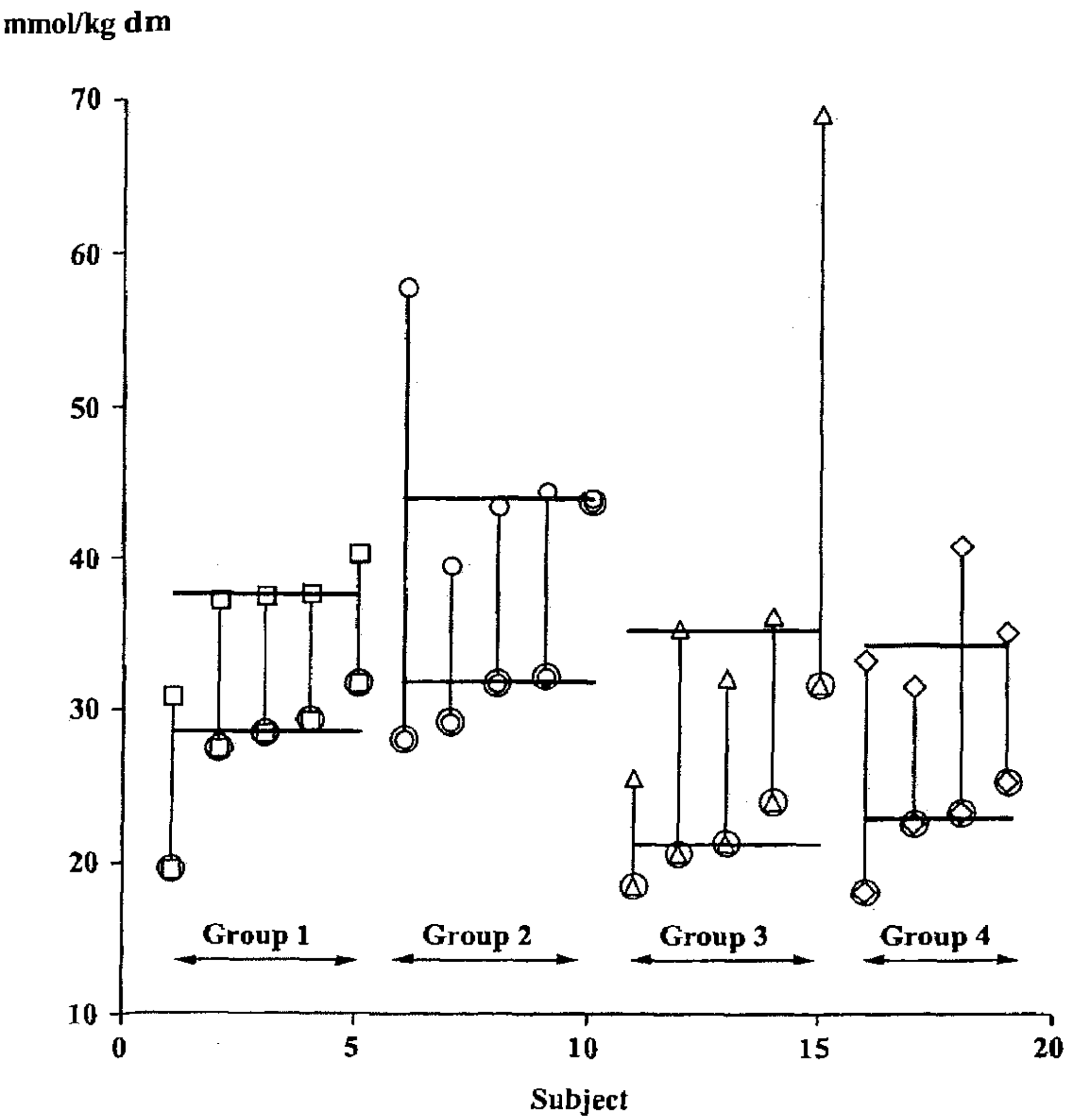

mmol/kg dm
70
60
50
40
30
20
10
0
5
10
15
20
Group 1
Group 2
Group 3
Group 4
Subject

Figure 13

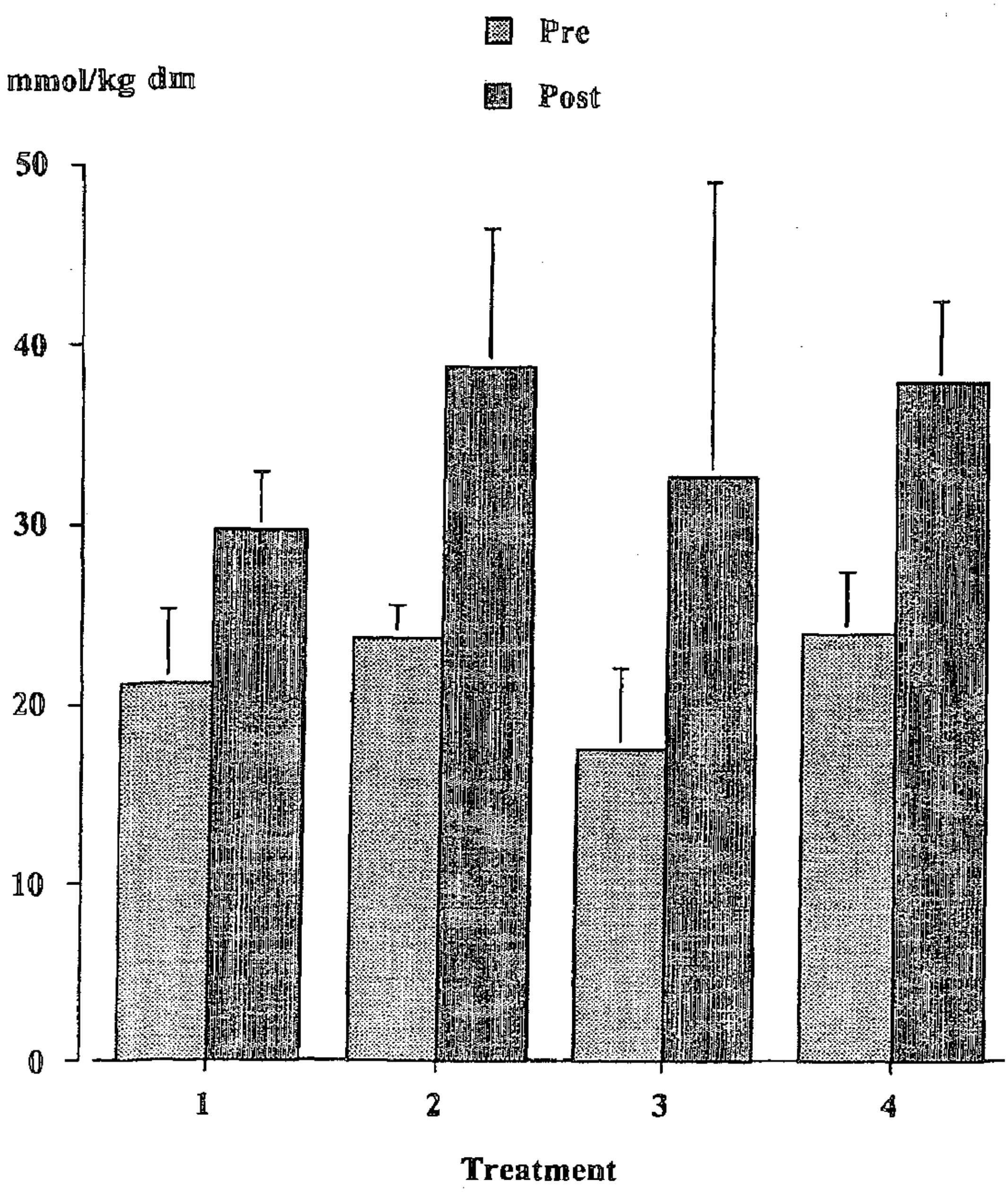


Figure 14

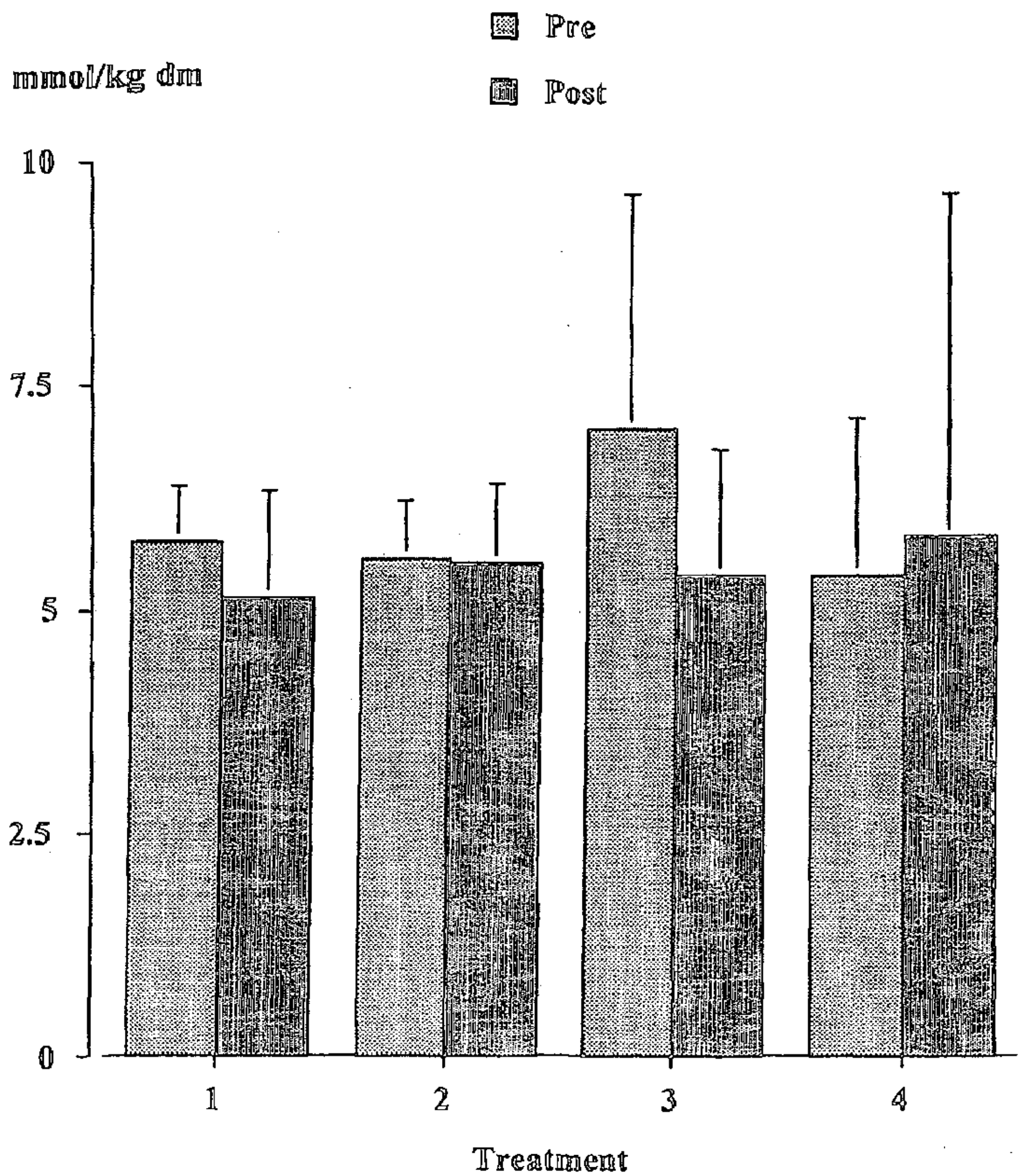


Figure 15

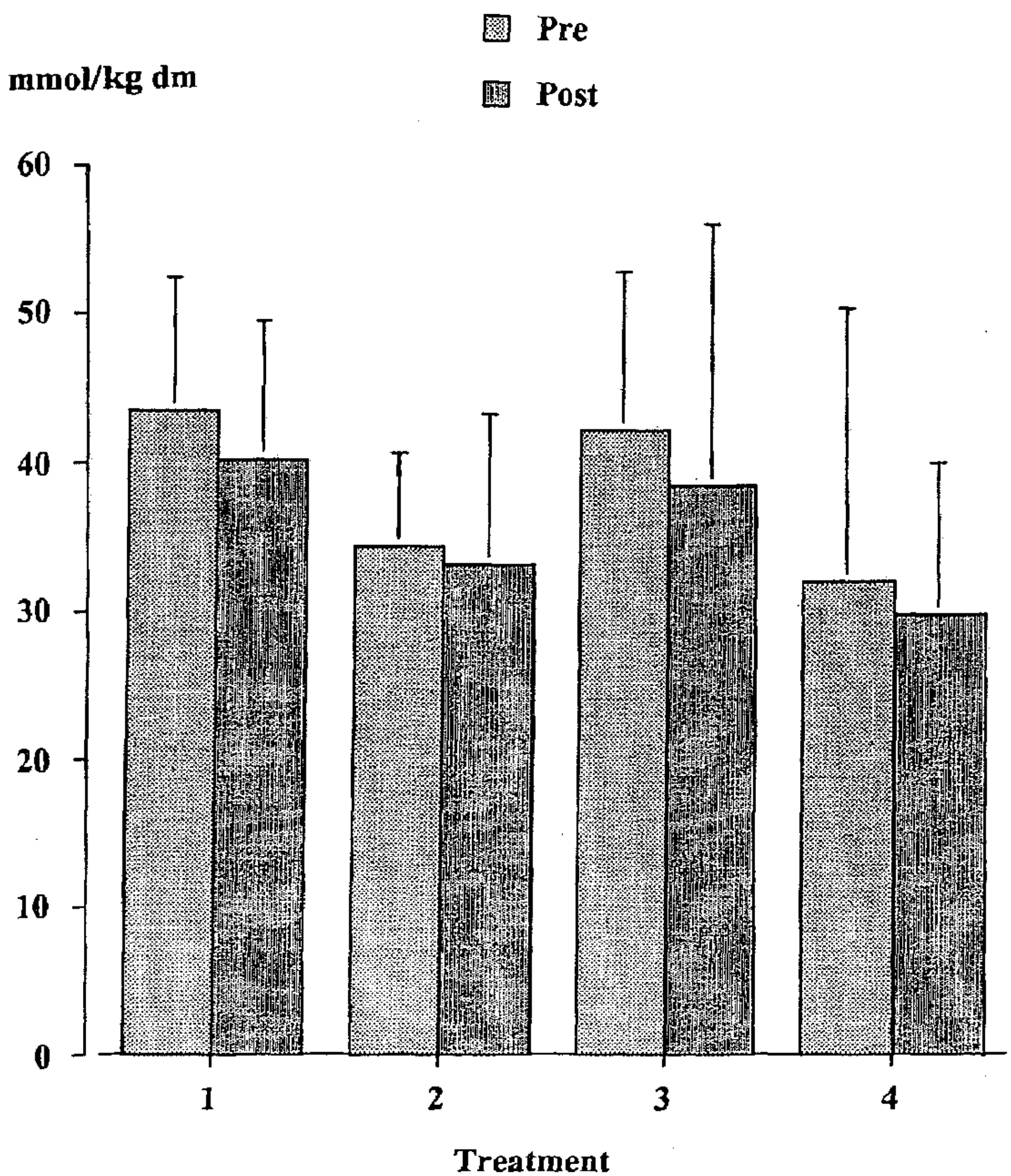
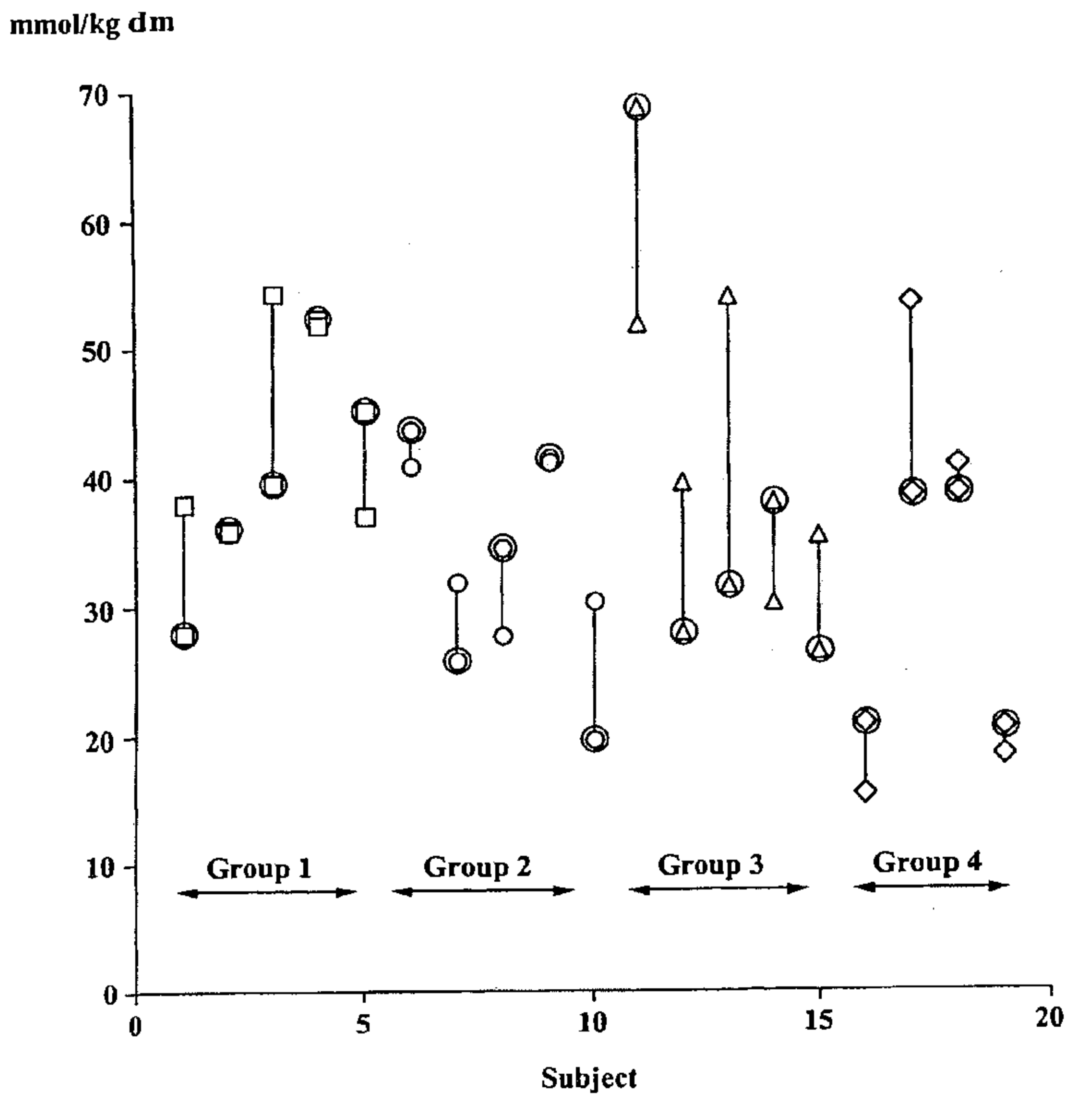


Figure 16



mmol/kg dm
70
60
50
40
30
20
10
0
Group 1
Group 2
Group 3
Group 4
0
5
10
15
20
Subject

Figure 17

Table 9

TREAT-MENT			DOSING TIMES								AVG DOSE (mg x times)	PER DAY	
			9am	10am	11am	12noon	3pm	4pm	5pm	6pm		GIVEN	as ß-Ala
1 Beta alanine (ß-Ala) n = 5	Week	1		800mg		800mg		800mg		800mg	800 x 4	3.2g	3.2g
		2		800mg		800mg		800mg		800mg	800 x 4	3.2g	3.2g
		3		800mg		800mg		800mg		800mg	800 x 4	3.2g	3.2g
		4		800mg		800mg		800mg		800mg	800 x 4	3.2g	3.2g
											Total 90g ß-Ala in 4W		
2 Beta alanine (ß-Ala) n = 5	Week	1	800mg	400mg	400mg	400mg	800mg	400mg	400mg	400mg	500 x 8	4.0g	4.0g
		2	800mg	400mg	400mg	800mg	800mg	400mg	400mg	800mg	600 x 8	4.8g	4.8g
		3	800mg	400mg	800mg	800mg	800mg	400mg	800mg	800mg	700 x 8	5.6g	5.6g
		4	800mg	800mg	800mg	800mg	800mg	800mg	800mg	800mg	800 x 8	6.4g	6.4g
											Total 146g ß-Ala in 4W		
3 Carnosine (C) n = 5	Week	1	1500mg	1500mg	1000mg	1000mg	1500mg	1500mg	1000mg	1000mg	1250 x 8	10g	4.0g
		2	1500mg	1500mg	1500mg	1500mg	1500mg	1500mg	1500mg	1500mg	1500 x 8	12g	4.8g
		3	2000mg	1500mg	1500mg	2000mg	2000mg	1500mg	1500mg	2000mg	1750 x 8	14g	5.6g
		4	2000mg	2000mg	2000mg	2000mg	2000mg	2000mg	2000mg	2000mg	2000 x 8	16g	6.4g
											Total 364g C in 4W (145g ß-Ala)		

METHODS AND COMPOSITIONS FOR INCREASING THE ANAEROBIC WORKING CAPACITY IN TISSUES

RELATED APPLICATIONS

This application claims the benefit of priority under 35 U.S.C. Section 119(e) of U.S. Provisional Application No. 60/462,238, filed Apr. 10, 2003, which is incorporated by reference herein.

The following applications also are incorporated by reference herein: U.S. application Ser. No. 10/209,169, filed Jul. 30, 2002; U.S. application Ser. No. 09/757,782, filed Jan. 9, 2001, now U.S. Pat. No. 6,426,361; U.S. application Ser. No. 09/318,530, filed May 25, 1999, now U.S. Pat. No. 6,172,098; U.S. application Ser. No. 08/909,513, filed Aug. 12, 1997, now U.S. Pat. No. 5,965,596; and United Kingdom application Nos. 9621914.2, filed Oct. 21, 1996, and 9616910.7, filed Aug. 12, 1996.

TECHNICAL FIELD

This invention relates to the fields of pharmaceuticals and physiology. In one aspect, the invention provides methods for increasing the buffering capacity of muscles and decreasing muscle fatigue. The invention also provides methods and compositions for increasing the anaerobic working capacity of muscle and other tissues.

BACKGROUND

Natural food supplements are typically designed to compensate for reduced levels of nutrients in the modern human and animal diet. In particular, useful supplements increase the function of tissues when consumed. It can be particularly important to supplement the diets of particular classes of animals whose normal diet may be deficient in nutrients available only from meat and animal products (e.g., human vegetarians and other animals who consume an herbivorous diet).

For example, in the sporting and athletic community, natural food supplements which specifically improve athletic ability are increasingly important, such as supplements that promote or enhance physical prowess for leisure or employment purposes. In another example, anaerobic (e.g., lactate-producing) stress can cause the onset of fatigue and discomfort that can be experienced with intense exercise (e.g., continuous or intermittent sprinting in soccer or ice-hockey), where oxygen availability may be limited (e.g., peripheral vascular disease, free diving or synchronized swimming) and with aging. Anaerobic stress can also result from prolonged submaximal isometric exercise when the local circulation is partially or totally occluded by the increase in intra-muscular pressure (e.g., during rock climbing). Excessive lactate production can result in the acidification of the intracellular environment.

Creatine (i.e., N-(aminoiminomethyl)-N-glycine, N-amidinosarcosine, N-methyl-N-guanylglycine, or methylglycocyamine) is found in large amounts in skeletal muscle and other "excitable" tissues (e.g., smooth muscle, cardiac muscle, or spermatozoa) characterized by a capacity for high and variable energy demand. Creatine is converted into phosphorylcreatine in energy-generating biochemical pathways within cells. In mammalian skeletal muscle, the typical combined content of creatine (i.e., creatine and phosphorylcreatine) may vary from less than 25 to about 50 mmol per kilogram fresh muscle (i.e., 3.2 to 6.5 grams per kilogram fresh muscle).

Creatine is formed in the liver and taken up into tissues, such as muscle, by means of an active transport system. Creatine synthesis in the body may also be augmented by the ingestion of creatine present in meat (e.g., 5-10 milligrams per kilogram body weight per day in the average meat-eating human and approximately zero in a vegetarian diet).

During sustained intense exercise, or exercise sustained under conditions of local hypoxia, the accumulation of hydronium ions formed during glycolysis and the accumulation of lactate (anaerobic metabolism) can severely reduce the intracellular pH. The reduced pH can compromise the function of the creatine-phosphorylcreatine system. The decline in intracellular pH can affect other functions within the cells, such as the function of the contractile proteins in muscle fibers.

Dipeptides (also referred to herein as peptides) of beta-alanine and histidine, and their methylated analogues, which include carnosine (beta-alanyl-L-histidine), anserine (beta-alanyl-L-1-methylhistidine), or balenine (beta-alanyl-L-3-methylhistidine), are present in the muscles of humans and other vertebrates. Carnosine is found in appreciable amounts in muscles of, for example, humans and equines. Anserine and carnosine are found in muscles of, for example, canines, camelids and numerous avian species. Anserine is the predominant beta-alanylhistidine dipeptide in many fish. Balenine is the predominant beta-alanylhistidine dipeptide in some species of aquatic mammals and reptiles. In humans, equines, and camelids, the highest concentrations of the beta-alanylhistidine dipeptides are found in fast-contracting glycolytic muscle fibers (type IIA and IIB) which are used extensively during intense exercise. Lower concentrations are found in oxidative slow-contracting muscle fibers (type I). See, e.g., Dunnett, M. & Harris, R. C. Equine Vet. J., Suppl. 18, 214-217 (1995). It is known that carnosine contributes to hydronium ion buffering capacity in different muscle fiber types, and up to 50% of the total in equine type II fibers.

SUMMARY

The invention provides methods of increasing anaerobic working capacity in a tissue, comprising the following steps: (a) providing a beta-alanylhistidine dipeptide and a glycine, an insulin, an insulin mimic, or an insulin-action modifier; and (b) administering the beta-alanine and at least one of the glycine, insulin mimic, or insulin-action modifier to the tissue in an amount effective to increase beta-alanylhistidine dipeptide synthesis in the tissue, thereby increasing the anaerobic working capacity in the tissue. The invention provides methods of regulating hydronium ion concentrations in a tissue comprising the following steps: (a) providing a beta-alanylhistidine dipeptide and a glycine, an insulin an insulin mimic, or an insulin-action modifier; and (b) administering the beta-alanine and at least one of the glycine, insulin mimic, or insulin-action modifier to the tissue in an amount effective to increase the hydronium ion concentration in the tissue.

In one aspect of the methods, the step of administering the beta-alanine and at least one of the glycine, insulin mimic, or insulin-action modifier to the tissue comprises oral administration, administration to a blood or blood plasma or a combination thereof. The beta-alanylhistidine dipeptide can comprise a carnosine, an anserine, or a balenine, or analogs or mimetics thereof.

The invention provides compositions comprising a mixture of a glycine, an insulin, an insulin mimic or an insulin-action modifier, and a composition comprising an amino acid or an active derivative thereof selected from the group consisting of a beta-alanine, a chemical derivative of beta-alanine and a peptide comprising a beta-alanine or analogs thereof. In one

aspect, the beta-alanine comprises a beta-alanylhistidine dipeptide, such as a carnosine, an anserine or a balenine or analogs thereof. The compositions can further comprise at least a creatine or a carbohydrate.

In one aspect, the insulin mimic comprises a D-pinitol (3-O-methyl-chiroinositol), a 4-hydroxy isoleucine, a demethyl-asterriquinone B-1 compound, an alpha lipoic acid, a R-alpha lipoic acid, a guanidiniopropionic acid, a vanadium compound, a vanadium complex or a synthetic phosphoinositolglycan peptide. The insulin-action modifier can be a sulphonylurea, a thiazolidinedione or a biguanide.

In alternative aspects, the composition is a pharmaceutical composition, a dietary supplement or a sports drink. The dietary supplement or sports drink can he a supplement for humans. The pharmaceutical composition can be formulated for humans.

The invention provides compositions comprising at least 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 or more grams of a peptide or an ester comprising a beta-alanine or analogs or mimetics thereof. The invention provides compositions comprising at least about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.1, 6.2, 6.3, 6.4, 6.5 or more grams of a peptide or an ester comprising a beta-alanine (or analogs or mimetics thereof) in an injectable form. In one aspect, the peptide comprises a beta-alanylhistidine dipeptide, such as a carnosine, an anserine or a balenine, or analogs or mimetics thereof.

The invention provides compositions formulated for humans comprising at least 200, 225, 250, 275, 300, 325, 350, 400, 425, 450, 475, 500, 525, 550, 575, 600, 625, 650, 675, 700, 750, 775, 800, 825, 850, 875, 900, 925, 950, 975 or 1000 or more mg of a heta-alanine or beta-alanine analogs or mimetics. In one aspect, the composition is formulated in an ingestible or an injectable formulation. The ingestible formulation can be a drink, a gel, a food or a tablet. The peptide can comprise a beta-alanylhistidine dipeptide, such as a carnosine, an anserine or a balenine, or analogs or mimetics thereof.

The invention provides methods of increasing the anaerobic working capacity of a tissue in a subject comprising the following steps: (a) providing a composition comprising (i) a mixture of a glycine, an insulin, an insulin mimic or an insulin-action modifier, and a composition comprising an amino acid or an active derivative thereof selected from the group consisting of a beta-alanine, a chemical derivative of beta-alanine and a peptide comprising a beta-alanine, or analogs or mimetics thereof; (ii) at least 0.5 gram of a peptide or an ester comprising a beta-alanine in an injectable form; or, (iii) at least 200 mg of a beta-alanine; and (b) administering the composition to the subject in an amount effective to increase the anaerobic working capacity of the tissue. In one aspect, the total dosage of the beta-alanine for a 24-hour period is at least about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.1, 6.2, 6.3, 6.4, 6.5 or more grams. The total dosage of the beta-alanine for a 24-hour period can be between about 0.2 gram and about 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7.0 or more grams. The composition can be given over a period of at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 or more days. The composition can be given over a period of at least ahout 3 days to about two, three, four or more weeks. The heta-alanine can comprise a beta-alanylhistidine dipeptide, such as a carnosine, an anserine or a balenine, or analogs or mimetics thereof. The total dosage of the beta-alanylhistidine dipeptide over a 24 hour period can be at least about 0.5 gram, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.1, 6.2, 6.3, 6.4, 6.5 or more grams. The total dosage of the beta-alanylhistidine dipeptide over a 24 hour period can be greater than about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 or more grams. The total dosage of the beta-alanylhistidine dipeptide over a 24 hour period can be more than about 5 gram to about 16 gram. The composition can be administered in multiple doses. The composition can be administered at least two times to eight times in a 24-hour period. In one aspect, about 200 mg, 225, 250, 275, 300, 325, 350, 400, 425, 450, 475, 500, 525, 550, 575, 600, 625, 650, 675, 700, 750, 775, 800, 825, 850, 875, 900, 925, 950, 975 or 1000 mgm of a beta-alanine (or analogs or mimetics thereof) and/or about 500 mg (or, about 200 mg, 225, 250, 275, 300, 325, 350, 400, 425, 450, 475, 500, 525, 550, 575, 600, 625, 650, 675, 700, 750, 775, 800, 825, 850, 875, 900, 925, 950, 975 or 1000 mgm) of carnosine (or analogs or mimetics thereof) is administered about two to eight, or more, times a day (e.g., 2, 3, 4, 5, 6, 7, 8 or more times a day) over a period of several weeks. In one aspect, at least about 2 g of a beta-alanine or at least about 5 g of carnosine is administered about two to eight times a day over a period of about two, three or four days.

In one aspect, the amount of a composition of the invention administered is increased daily. The amount of the composition of the invention administered can be increased weekly. The composition can be administered in treatment periods that last for at least about four weeks.

While the invention is not limited by any particular mechanism of action, the invention provides methods of regulating hydronium ion concentration in tissue in a subject comprising the following steps: (a) providing a composition comprising (i) a mixture of a glycine, an insulin, an insulin mimic or an insulin-action modifier, and a composition comprising an amino acid or an active derivative thereof selected from the group consisting of a beta-alanine, a chemical derivative of beta-alanine and a peptide comprising a beta-alanine or analogs or mimetics thereof; (ii) at least 0.5 gram of a peptide or an ester comprising a beta-alanine in an injectable form; or, (iii) at least 200 mg of a beta-alanine; and (b) administering the composition to the subject in an amount effective to regulate the hydronium ion concentration in the tissue.

In one aspect, the invention features methods and compositions for increasing the anaerobic working capacity of muscle and other tissues. The methods and compositions of the invention provide for the simultaneous accumulation of creatine and/or beta-alanylhistidine dipeptides, or beta-alanine and L-histidine analogues, within a tissue in the body. The methods include ingesting or infusing compositions into the body. In one aspect, the compositions are mixtures of compounds capable of increasing the availability and uptake of creatine and of precursors for the synthesis and accumulation of beta-alanylhistidine dipeptides in human and animal tissue. The compositions of the invention can induce the synthesis and accumulation of beta-alanylhistidine dipeptides in a human or animal body when introduced into the body.

The compositions can include beta-alanine, chemical derivatives and analogs of beta-alanine such as esters of beta-alanine, peptides of beta-alanine, such as carnosine, anserine, and balenine, as well as analogues thereof. The compositions may also include L-histidine and mixtures thereof. Each of the beta-alanine and/or L-histidine can he formulated or administered as individual amino acids, or, as components of dipeptides (e.g., carnosine, anserine, and/or balenine), oligopeptides, or polypeptides. The beta-alanine, L-histidine, carnosine, anserine, and/or balenine, or peptides of beta-alanine can be active derivatives. An active derivative is a compound derived from, or is a precursor of, a substance and

performs in the same or similar way in the body as the substance, or which is processed into the substance when placed into the body. Examples include, for example, esters and amides.

Compositions can also include any one or more of a creatine, a carbohydrate, insnlin, an insulin mimic, an insulin-action modifier or a glycine. The compositions of the invention can be used for the preparation of a dietary supplement (including, e.g., drinks, gels, foods) or pharmaceutical composition for humans or animals. The compositions of the invention can be used in any of the methods of the invention.

In one aspect, the invention features compositions for and a method of regulating hydronium ion concentrations in a tissue. The method includes the steps of providing an amount of beta-alanine to blood or blood plasma effective to increase beta-alanylhistidine dipeptide synthesis in a tissue and exposing the tissue to the blood or blood plasma, whereby the concentration of beta-alanylhistidine is increased in the tissue. The beta-alanylhistidine may be a carnosine, anserine, or a balenine. The method can include the step of providing an amount of L-histidine to the blood or blood plasma effective to increase beta-alanylhistidine dipeptide synthesis.

In another aspect, the invention features a method of increasing the anaerobic working capacity of a tissue. The method includes the steps of providing an amount of beta-alanine to blood or blood plasma effective to increase beta-alanylhistidine dipeptide synthesis in a tissue, providing an amount of L-histidine to the blood or blood plasma effective to increase beta-alanylhistidine dipeptide synthesis in a tissue, and exposing the tissue to the blood or blood plasma. The concentration of beta-alanylhistidine is increased in the tissue.

In alternative aspects, the methods can include the step of increasing a concentration of creatine in the tissue. The increasing step can include providing an amount of creatine to the blood or blood plasma effective to increase the concentration of creatine in the tissue (e.g., by providing creatine to the blood or blood plasma).

The providing steps of the methods can include ingestion, infusion (e.g., injection) or a combination of ingestion and infusion, of a composition including an amount of beta-alanine, a peptide of beta-alanine such as carnosine, anserine and balenine which are hydrolyzed to their constituent amino acids on ingestion and are a source of beta-alanine for the body. Methods of the invention also include providing L-histidine, creatine, carbohydrate, insulin, insulin mimics, insulin-action modifiers and/or glycine.

In yet another aspect, the methods can include increasing a concentration of insulin in the blood or blood plasma. The concentration of insulin can be increased, for example, by injection of insulin. Methods of the invention can also include injection ingestion, or other modes of delivery, known to those of skill in the art, to a body (also referred to as a subject) of insulin mimics. Examples of insulin mimics include, but are not limited to, D-pinitol (3-O-methyl-chiroinositol), 4-hydroxy isoleucine, L783,281 (a demethyl-asterriquinone B-1 compound), alpha lipoic acid, R-alpha lipoic acid, guanidiniopropionic acid, vanadium compounds such as vanadyl sulfate or vanadium complexes such as peroxovanadium, and synthetic phosphoinositolglycans (PIG peptides). Additionally or alternatively, methods of the invention can include the use of insulin-action modifiers to enhance or inhibit the action of insulin in the body. Examples of insulin-action modifiers can include, but are not limited to, sulphonylureas, thiazolidinediones, and biguanides.

In still another aspect, the methods include providing glycine to a body. It is thought that glycine may suppress blood glucose release in the blood after ingestion of a meal. It may be that glycine enhances insulin sensitivity by promoting greater glucose uptake. Accordingly, the methods include providing glycine alone or in conjunction with insulin, insulin mimics or insulin-action modifiers in the compositions and methods of the invention. Glycine may be provided in various forms, for example, alone or in combination with other substances, such as in dietary supplements. Alternatively, glycine can be derived from other sources, sucb as gelatin.

The tissue referred to in the invention can be a skeletal muscle.

In one aspect, the invention provides compositions for practicing the methods of the invention. Accordingly, one aspect of the invention contemplates a composition having one or more active ingredient, including beta-alanine, beta-alanylhistidine peptides (or analogues or derivatives thereof), creatine, insulin, insulin mimics or insulin-action modifiers, glycine, and carbobydrate, to carry out tbe methods of the invention. The invention further contemplates the use of multiple compositions formulated to provide one or more active ingredient to the body for carrying out the methods of the invention.

Therefore, in an exemplary aspect, the invention features a composition consisting essentially of beta-alanine or a peptide source of beta-alanine, between about 39 and about 99 percent by weight of a carbohydrate, and up to about 60 percent by weight of water. The composition can include between about 0.1 and about 20 percent by weight of the beta-alanine (in the free or a bound form). The composition can include between about 0.1 and about 20 percent by weight of L-histidine.

The carbohydrate can be a simple carbohydrate (e.g., glucose).

In another aspect, the invention features a composition consisting essentially of beta-alanine or a peptide source of beta-alanine, between about 1 and about 98 percent by weight of a creatine source, and up to about 97 percent by weight of water. The composition includes between about 0.1 and about 98 percent by weight of the beta-alanine. The peptide source can include L-histidine and the composition can include between about 0.1 and about 98 percent by weight of L-histidine from this source.

The peptide source can be a mixture of amino acids, dipeptides, oligopeptides, polypeptides, or active derivatives thereof.

The composition can be a dietary supplement. The creatine source can be creatine monohydrate.

The concentrations of components in blood or blood plasma, including beta-alanine, can be increased by infusion (i.e., injection) or ingestion of an agent operable to cause an increase in the blood plasma concentration. The composition can be ingested in doses of between about 100 milligrams and about 800 grams or more per day. The doses can be administered in one part or multiple parts each day.

An increase of creatine and beta-alanylhistidine dipeptides in the muscles can increase the tolerance of the cells to an increase in hydronium ion production with anaerobic work and lead to an increase in endurance during exercise before the onset of fatigue. The compositions and methods can contribute to correcting the loss of beta-alanine, L-histidine, or creatine due to degradation or leaching of these constituents during the cooking or processing of food. The compositions and methods can also contribute to correcting the absence of these components from a vegetarian diet.

The methods and compositions can be used to increase beta-alanylhistidine dipeptides in sportsmen, athletes, body-builders, synchronized swimmers, soldiers, elderly people, horses in competition, working and racing dogs, and game birds, to avoid or delay the onset of muscular fatigue.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other advantages and features of the invention will be apparent to the skilled artisan from the detailed description, drawings, and claims.

All publications, patents, patent applications cited herein are hereby expressly incorporated by reference for all purposes.

DESCRIPTION OF DRAWINGS

FIG. **1** is a graph depicting changes in the concentrations of beta-alanine in blood plasma of five horses, before and at 2 hour intervals following the feeding of beta-alanine and L-histidine (100 milligrams per kilogram body weight and 12.5 milligrams per kilogram body weight, respectively, three times per day) over a period of 30 days.

FIG. **2** is a graph depicting changes in the concentrations of L-histidine in blood plasma of five horses, before and at 2 hour intervals following the feeding of beta-alanine and L-histidine (100 milligrams per kilogram body weight and 12.5 milligrams per kilogram body weight, respectively, three times per day) over a period of 30 days.

FIGS. **3***a*, **3***b*, **3***c*, **3***d*, **3***e* and **3***f* are graphs depicting the contrast in the changes in the concentrations of beta-alanine in blood plasma of six horses, before and at hourly intervals following the feeding of beta-alanine and L-histidine, as described in detail, below.

FIGS. **4***a*, **4***b*, **4***c*, **4***d*, **4***e* and **4***f* are graphs depicting the contrast in the changes in the concentrations of L-histidine in blood plasma of six horses, before and at hourly intervals following the feeding of beta-alanine and L-histidine, as described in detail, below.

FIG. **5** is a graph depicting the contrast in the changes in the mean concentrations of beta-alanine in equine blood plasma (n=6), before and at hourly intervals following the feeding of beta-alanine and L-histidine, as described in detail, below.

FIG. **6** is a graph depicting the contrast in the changes in the mean concentrations of L-histidine in equine blood plasma (n=6), before and at hourly intervals following the feeding of beta-alanine and L-histidine (100 milligrams per kilogram body weight and 12.5 milligrams per kilogram body weight, respectively, three times per day) on the first and last day of a 30 day period of dietary supplementation.

FIG. **7** is a graph depicting the correlation between the increase in 6 thoroughbred horses in the carnosine concentration in type II skeletal muscle fibers (the average of the sum of type IIA and IIB fibers) and the increase, between the 1st and 30th day of supplementation, in the area under the blood plasma beta-alanine concentration-time curve over the first 12 hours of the day ($AUC_{(0\text{-}12\ hr)}$).

FIG. **8** is graph depicting the mean results of the administration of beta-alanine, broth, or carnosine to test subjects.

FIG. **9** is a graph depicting mean changes in plasma beta-alanine over nine hours of treatment.

FIG. **10** is a graph depicting the mean changes in plasma beta-alanine over 9 hours following the oral ingestion of 10 milligrams per kilogram body weight of beta-alanine.

FIG. **11** is a graph depicting the mean (n=6) plasma beta-alanine concentration over the 24 hours of Day 1 and Day 30 of the treatment period.

FIG. **12** is a graph depicting changes in muscle carnosine concentration pre and post treatment in different subjects. The red circles indicate the muscle concentrations prior to supplementation.

FIG. **13** is a graph depicting muscle concentration (mean+SD) of carnosine before and post supplementation in three different treatment groups.

FIG. **14** is a graph depicting muscle concentration (mean+SD) of histidine before and post supplementation in three different treatment groups.

FIG. **15** is a graph illustrating data showing muscle concentration (mean+SD) of taurine before and post supplementation in four different treatment groups.

FIG. **16** is a graph illustrating data showing muscle concentration (mean+SD) of taurine before and post supplementation in different subjects.

FIG. **17** illustrates a table of data, described in detail as Table 9, below.

Like reference symbols in the various drawings indicate like elements.

DETAILED DESCRIPTION

The invention provides compositions comprising beta-alanine, peptides of beta-alanine, analogues and derivatives thereof, beta-alanylhistidine dipeptides (e.g., carnosine, anserine, and balenine) and methods using these compositions for increasing the anaerobic working capacity of a tissue comprising providing an amount of beta-alanine to blood or blood plasma effective to increase beta-alanylhistidine dipeptide synthesis in a tissue. Beta-alanylhistidine dipeptides can include peptides of beta-alanine, such as carnosine, anserine, and balenine. In one aspect, they can have pKa values between approximately 6.8 and 7.1. In one aspect, they can be involved in the regulation of intra-cellular pH homeostasis during muscle contraction and the development of fatigue. The content of other substances involved in hydronium ion buffering, such as amino acid residues in proteins, inorganic and organic phosphates and bicarbonate, can be constrained by their involvement in other cell functions. In one aspect, the beta-alanylhistidine dipeptides provide an effective way of accumulating pH-sensitive histidine residues into a cell. Variations in the muscle beta-alanylhistidine dipeptide concentrations affect the anaerobic work capacity of individual athletes.

The beta-alanylhistidine dipeptides are synthesized within the body from beta-alanine and L-histidine. These precursors can be generated within the body or are made available via the diet, including from the breakdown of an ingested beta-alanylhistidine dipeptide. Within the body, beta-alanine is transported to tissues such as muscle. In a typical fed state, the concentration of beta-alanine is low in comparison with the concentration of L-histidine in human and equine blood plasma. These concentrations should be viewed in relation to the affinity of the carnosine synthesizing enzyme, carnosine synthetase, for its substrates as determined by the Michaelis-Menton constant (Km). The Km for histidine is about 16.8 μM. The Km for beta-alanine is between about 1000 and 2300 μM. The low affinity of carnosine synthetase for beta-alanine, and the low concentration of beta-alanine in muscle, demonstrate that the concentration of beta-alanine in muscle is limiting to the synthesis of the beta-alanylhistidine dipeptides.

Increasing the amount of beta-alanylhistidine dipeptides within a muscle favorably affects muscular performance and the amount of work that can be performed by the muscle. Accordingly, it is desirable to increase the synthesis and accumulation of beta-alanylhistidine dipeptides in a tissue in a human or animal body.

The synthesis and accumulation of beta-alanylhistidine dipeptides in a human or animal body can be increased by increasing creatine within the body, increasing the blood or blood plasma concentrations of beta-alanine, increasing the blood or blood plasma concentrations of beta-alanine and creatine, or increasing the blood or blood plasma concentrations of beta-alanine, L-histidine, and creatine. The increase in dipeptides can be simultaneous with the increase in beta-alanine concentrations.

In one aspect, the compositions and methods of the invention can be used to increase blood plasma concentrations of beta-alanine, L-histidine and/or creatine by ingestion or infusion of beta-alanine, peptides of beta-alanine, L-histidine, creatine, carnosine, anserine, and/or balenine and/or active derivatives or analogs thereof alone or in various combinations. The compositions of the invention can be administered orally, enterally, or parenterally. For example, compositions of the invention can be orally ingested or infused through the skin through a topical cream or a patch.

The composition can include carbohydrates (e.g., simple carbohydrates), insulin, or agents that stimulate the production of insulin. Compositions can also include glycine, insulin, insulin mimics, and/or insulin-action modifies.

The compositions can be a dietary supplement that can be ingested, injected, or absorbed through the skin. Preferably, the compositions can be administered in one or more doses per day. The beta-alanine dosage can be between about 1 milligram and about 200 milligrams per kilogram body weight or the dose of a peptide of beta-alanine (e.g. carnosine) from 2.5 milligrams to 500 milligrams per kilogram body weight. In one aspect, the total amount of beta-alanine (or other composition of the invention) administered can be at least 200 mg, from 200 mg to 5 g, or from 5 g or more per day for a human. A single dose of active ingredient, e.g., beta-alanine, carnosine, anserine, or balenine, or mixtures thereof, may be formulated to be in the amount about 200, 400, 800 mg or more. The creatine (e.g., creatine monohydrate) dosage, or dosage of other compositions of the invention, can be between about 5 milligrams to 200 milligrams per kilogram body weight. The L-histidine dosage, or dosage of other compositions of the invention, can be between about 1 milligram to 100 milligrams per kilogram body weight. The simple carbohydrate (e.g., glucose) dosage, or dosage of compositions of the invention, can be between about 0.5 and 2.0 grams per kilogram body weight.

In an 80 kilogram person, suitable dosages per day can be between 0.08 grams to 16.0 grams of beta-alanine or 200 milligrams to 40 grams of a peptide of beta-alanine, 0.4 grams to 16.0 grams of creatine monohydrate, 0.08 grams to 8.0 grams of L-histidine, or 40 grams to 160 grams of glucose or other simple carbohydrate. The composition can be in a solid form or a liquid form or in a suspension which can be ingested or infused into the body. The composition is can be ingested by humans in an amount of between 0.08 grams and 1000 grams or more per day, which may be taken in one or more parts throughout the day. In animals, the daily intake will be adjusted by body weight.

In one aspect, the total amount of a peptide of beta-alanine, for example, carnosine, anserine or balenine that can be administered per day may be at least 500 mg, between about 500 mg to about 5 g, between about 5 g to about 16 g, or greater than 16 g. A single dose of a peptide of beta-alanine creatine, anserine or balenine, or mixtures thereof, may be formulated to be in the amount of 0.5, 1, 1.5, or 2 g (for each, or all, or the peptides in a formulation comprising a mixture).

For humans and animals, the compositions can be:

(a) 1% to 99% by weight of beta-alanine or 1% to 99% by weight of a peptide of beta-alanine;
 1% to 99% by weight of creatine monohydrate; and
 0% to 98% by weight of water;

(b) 1% to 98% by weight of beta-alanine or 1% to 98% by weight of a peptide of beta-alanine;
 1% to 98% by weight of L-histidine;
 1% to 98% by weight of creatine monohydrate; and
 0% to 97% by weight of water;

(c) 1% to 20% by weight of beta-alanine or 1% to 20% by weight of a peptide of beta-alanine;
 39% to 99% by weight of glucose or other simple carbohydrate; and
 0% to 60% by weight of water; or

(d) 1% to 20% by weight of beta-alanine or 1% to 20% by weight of a peptide of beta-alanine;
 1% to 20% by weight of L-histidine
 39% to 99% by weight of glucose or other simple carbohydrate; and
 0% to 60% by weight of water.

In one aspect, compositions are applied to a body for at least three days, from 3 days to 2 weeks, from 2 weeks to 4 weeks, or longer. In certain regimens, the daily dosages are gradually increased or decreased. This can be done daily, every couple of days, or weekly.

EXAMPLES

The following are specific examples of the methods and compositions for increasing the anaerobic working capacity of muscle and other tissues.

Example 1

The effect of supplementation of a normal diet with multiple daily doses of beta-alanine and L-histidine on the carnosine concentration in type I, IIA, and IIB skeletal muscle fibers of thoroughbred horses was assessed. Six experimental thoroughbred horses of normal health (three fillies and three geldings), aged 4 to 9 years, underwent one month (30 days) of dietary conditioning (pre-supplementation period) prior to the commencement of the supplementation period. During the dietary conditioning period each horse was fed a diet comprising 1 kilogram of pelleted feed (Spillers racehorse cubes) and 1 kilogram of soaked sugar beet pulp as a source of complex and simple carbohydrates, three times per day (at 08:30, 12:30, and 16:30, respectively). Soaked hay (3 kilograms dry weight) was also provided twice daily (at 09:00 and 17:00). Water was provided ad libitum.

During the supplementation period, an identical feeding regime was implemented. However, each hard feed meal was supplemented with beta-alanine and L-histidine (free base). Beta-alanine and L-histidine were mixed directly into the normal feed. Individual doses of beta-alanine and L-histidine were calculated according to body weight. Beta-alanine was administered at 100 milligrams per kilogram body weight and L-histidine at 12.5 milligrams per kilogram body weight. Dietary supplementation was begun on day 1 of the protocol

and discontinued at the end of day 30. Heparinized blood samples (5 milliliters) were collected on days 1, 6, 18, 24, and 30. On day 1 and day 30, blood samples were collected prior to the first feed and at hourly intervals for a total of 12 hours each day. On the three intervening sampling days, blood was collected prior to the first feed and 2 hours after each subsequent feed. On the day before the start of supplementation (day 0) a muscle biopsy was taken, following application of local anesthesia of the skin, from the right middle gluteal muscle (m. gluteus medius) of each horse using a Bergstrom-Stille percutaneous biopsy needle. Subsequent muscle biopsies were collected immediately after the end of the supplementation period (day 31) as close as possible to the original sampling site. Clinical monitoring of the horses was performed daily. This comprised a visual examination and measurement of body weight, twice-daily measurement of rectal temperature, and weekly blood sampling for clinical biochemistry and hematology. During the course of the study, the horses received no formal training or exercise, although they were allowed one hour of free exercise each day.

Fragments of individual muscle fibers dissected from freeze-dried muscle biopsies were characterized as either type I, IIA or IIB by histochemical staining for myosin ATPase activity at pH 9.6 following pre-incubation at pH 4.5 by a modification of the method described in, Kaiser and Brook, *Arch. Neurol.*, 23:369-379 (1970).

Heparinized blood plasma samples were extracted and analyzed for beta-alanine and L-histidine concentrations by high-performance liquid chromatography (HPLC). Individual weighed muscle fibers were extracted and analyzed for carnosine by HPLC according to the method described in, Dunnett and Harris, "High-performance liquid chromatographic determination of imidazole dipeptides, histidine, 1-methylhistidine and 3-methylhistidine in muscle and individual muscle fibers," *J. Chromatogr. B. Biomed. Appl.*, 688: 47-55 (1997).

Differences in carnosine concentrations within fiber types before and after supplementation were established within horses using one-way analysis of variance (ANOVA). In instances where differences were detected, significance was determined using a multiple comparison test (Fisher's PLSD).

No palatability problems were encountered with the addition of beta-alanine and L-histidine to the feed. No adverse physiological or behavioral effects of the supplemented diet were observed in any of the horses during the thirty days of supplementation. No significant changes in body weight were recorded, and rectal temperatures remained within the normal range. No acute or chronic changes in clinical biochemistry or hematology were observed. Beta-alanine was not detected in the plasma of any of the horses prior to the start of supplementation. The lower limit of quantitation for beta-alanine in plasma by the assay used was 3 micromolar (μM). Plasma L-histidine concentrations in the six horses prior to the start of supplementation were between 36.6 and 54.4 μM.

Individual changes in blood plasma beta-alanine and L-histidine concentrations for five of the six horses over all the sampling days are shown in FIGS. 1 and 2, respectively. There was a trend towards an increase in the pre-feeding concentrations of blood plasma beta-alanine and L-histidine with increasing time of supplementation. Furthermore, over the thirty day supplementation period, the blood plasma concentration response to supplementation was also increased. The response was greater for beta-alanine.

Comparisons of the changes in blood plasma beta-alanine and L-histidine concentrations prior to the first feed of the day, and hourly thereafter between the first and last days of the supplementation period, for the six individual horses, are shown in FIGS. 3*a* and 3*b*, and FIGS. 4*a* and 4*b*, respectively. FIGS. 3*a*, 3*b*, 3*c*, 3*d*, 3*e* and 3*f* are graphs depicting the contrast in the changes in the concentrations of beta-alanine in blood plasma of six horses, before and at hourly intervals following the feeding of beta-alanine and L-histidine (100 milligrams per kilogram body weight and 12.5 milligrams per kilogram body weight, respectively, three times per day) on the first and last day of a 30 day period of dietary supplementation. FIGS. 4*a*, 4*b*, 4*c*, 4*d*, 4*e* and 4*f* are graphs depicting the contrast in the changes in the concentrations of L-histidine in blood plasma of six horses, before and at hourly intervals following the feeding of beta-alanine and L-histidine (100 milligrams per kilogram body weight and 12.5 milligrams per kilogram body weight, respectively, three times per day) on the first and last day of a 30 day period of dietary supplementation. FIG. 5 is a graph depicting the contrast in the changes in the mean concentrations of beta-alanine in equine blood plasma (n=6), before and at hourly intervals following the feeding of beta-alanine and L-histidine (100 milligrams per kilogram body weight and 12.5 milligrams per kilogram body weight, respectively, three times per day) on the first and last day of a 30 day period of dietary supplementation. The mean (SD) changes (n=6) in blood plasma beta-alanine concentration over time during the 24 hours of the first (day 1) and last (day 30) days of the supplementation period are contrasted in FIG. 5. The area under the mean blood plasma beta-alanine concentration versus time curve over 24 hours ($AUC_{(0-24hr)}$) was much greater on day 30 of the supplementation.

The mean (SD) changes (n=6) in blood plasma L-histidine concentration over time during the 24 hours of the first (day 1) and last (day 30) days of the supplementation period are contrasted in FIG. 6. The area under the mean blood plasma beta-alanine concentration vs. time curve over 24 hours ($AUC_{(0-24hr)}$) was greater on day 30 of the supplementation. The greater AUC for blood plasma beta-alanine on the last day of supplementation (day 30) in contrast to the first day of supplementation (day 1) suggests the increased uptake of beta-alanine from the equine gastro-intestinal tract with progressive supplementation. A similar effect was observed for changes in blood plasma L-histidine concentration during the supplementation period. Peak blood plasma concentrations of beta-alanine and L-histidine occurred approximately one to two hours post-feeding in each case.

A total of 397 individual skeletal muscle fibers (192 pre-supplementation; 205 post-supplementation) from the six horses were dissected and analyzed for carnosine. Mean (SD) carnosine concentration, expressed as millimoles per kilogram dry weight ($mmol\ kg^{-}dw$), in pre- and post-supplementation type I, IIA, and IIB skeletal muscle fibers from the six individual horses are given in Table 1 where n is the number of individual muscle fibers analyzed. Following thirty days of beta-alanine and L-histidine supplementation the mean carnosine concentration was increased in type IIA and IIB fibers in all six horses. These increases were statistically significant in seven instances. The increase in mean carnosine concentration in type IIB skeletal muscle fibers was statistically significant in five out of six horses. The increase in mean carnosine concentration in type IIA skeletal muscle fibers was statistically significant in two out of six horses.

TABLE 1

Horse	Day	Type I	n	Type IIA	n	Type IIB	n
6	0	32.3 (14.5)	3	72.1 (47.7)	11	111.8 (22.8)	14
	31	—		16.2 (20.9)	17	117.7 (38.7)	12
5	0	59.5 (3.9)	2	102.6 (12.7)	12	131.2 (26.6)	26
	31	55.5	1	112.2 (17.1)	18	153.3 (28.0)**	22
4	0	44.8 (6.6)	4	59.9 (19.5)	13	108.6 (41.5)	19
	31	37.0 (9.3)	2	88.0 (34.2)*	17	152.4 (65.0)*	19
1	0	56.7 (5.3)	2	88.5 (20.9)	15	101.3 (15.2)	13
	31	57.8	1	96.1 (17.3)	19	14.3 (13.3)*	11
2	0	—		89.6 (16.2)	13	104.2 (22.2)	14
	31	65.9 (13.2)	4	102.2 (22.1)	18	142.0 (35.4)***	12
3	0	30.9 (4.0)	2	85.1 (20.3)	6	113.5 (20.4)	23
	31	—		105.0 (17.6)*	23	135.4 (24.9)*	9
Mean	0	44.8	13	83.0	70	111.8	109
	31	54.1	8	96.6*	112	135.9**	85

*significantly different to pre-supplementation, $p < 0.05$
**significantly different to pre-supplementation, $p < 0.01$
***significantly different to pre-supplementation, $p < 0.005$

The absolute (e.g. mmol kg^{-1} dw) and percentage increases in the mean carnosine concentrations in type IIA and IIB skeletal muscle fibers from the six horses are listed in Table 2.

TABLE 2

Horse	Type IIA Absolute increase	Type IIA % increase	Type IIB Absolute increase	Type IIB % increase
6	4.1	5.7	5.6	5.3
5	9.6	9.4	22.1	16.8
4	28.1	46.9	43.8	40.3
1	7.6	8.6	13.0	12.8
2	12.6	14.1	37.8	36.3
3	19.9	23.4	21.9	19.3
Mean	13.6	18.0	24.1	21.8

It was observed that the individual horses which showed the greater increase in muscle carnosine concentration following thirty days of supplementation also demonstrated the greater increase in blood plasma heta-alanine AUC between day 1 and day 30 of the supplementation period. Referring to FIG. 7, a significant correlation ($r=0.986$, $p<0.005$) for five of the six horses was observed between the increase in mean carnosine concentration, averaged between type IIA and IIB skeletal muscle fibers and the increase, between the 1st and 30th day of supplementation, in blood plasma beta-alanine AUC, over the first 12 hours($AUC_{(0\text{-}12\ hr)}$). Only five horses were used to calculate the regression line. Horse 6 (filled circle) showed no appreciable increase in blood plasma beta-alanine concentration greater than that observed on day 1 until the last day of supplementation. This was unlike the other five horses, which showed a progressive increase with each sampling day. For this reason horse 6 was excluded from the calculation of the regression equation.

Increases in muscle carnosine concentration following thirty days of supplementation with beta-alanine and L-histidine will cause a direct increase in total muscle buffering capacity. This increase can be calculated by using the Henderson-Hasselbach Equation. Calculated values for the increases in muscle buffering capacity in type IIA and IIB skeletal muscle fibers in the six thoroughbred horses are shown in Table 3.

TABLE 3

Horse	Day	Type IIA βmcar	Type IIA βmtotal	Type IIA Δβmtotal (%)	Type IIB βmcar	Type IIB βmtotal	Type IIB Δβmtotal (%)
6	0	23.9	93.9	+1.5	37.1	107.1	+1.8
	31	25.3	95.3		39.0	109.0	
5	0	34.0	104.0	+3.1	43.5	113.5	+6.4
	31	37.2	107.2		50.8	120.8	
4	0	19.9	89.9	+10.3	36.0	106.0	+13.7
	31	29.2	99.2		50.5	120.5	
1	0	29.3	99.3	+2.6	33.6	103.6	+4.2
	31	31.9	101.9		37.9	107.9	
2	0	29.7	99.7	+4.2	34.5	104.5	+12.1
	31	33.9	103.9		47.1	117.1	
3	0	28.2	98.2	+6.7	37.6	107.6	+6.8
	31	34.8	104.8		44.9	114.9	
Mean	0	27.5	97.5	+4.7	37.1	107.1	+7.5
	31	32.1	102.1		45.0	115.0	

Example 2

The effect of supplementation of a normal diet with single and multiple daily doses of beta-alanine in free or peptide bound form on the beta-alanine and beta-alanyl dipeptide concentrations of plasma of humans was assessed. The plasma concentration of beta-alanine in six normal subjects following the consumption of a broth delivering approximately 40 milligrams per kilogram body weight of beta-alanine was monitored. Doses of 10 and 20 milligrams per kilogram body weight of beta-alanine were also given.

2.5 milliliter venous blood samples were drawn through an indwelling catheter at 10 minute intervals for the first 90 minutes and then after 120, 180, 240 and 360 minutes. The blood samples were dispensed into tubes containing lithium-heparin as an anti-coagulant. The catheter was maintained by flushing with saline. Plasma samples were analyzed by HPLC according to the method described in Jones & Gilligan (1983) *J. Chromatogr.* 266:471-482 (1983).

Table 4 summarizes the allocation of treatments during the beta-alanine absorption study. The estimated equivalent doses of beta-alanine are presented in Table 3.

TABLE 4

Subject	Age yrs	Weight kg	Broth 40 mg/kg bwt	β-ala 0 mg/kg bwt	β-ala 10 mg/kg bwt	β-ala 20 mg/kg bwt	β-ala 40 mg/kg bwt	Carnosine 20 mg/kg bwt
1	53	76	+			+	+	+
2	33	60	+			+	+	
3	29	105	+	+	+	+		
4	31	81	+	+	+		+	
5	30	94	+	+	+		+	
6	25	65	+	+	+	+		

The broth was prepared as follows. Fresh chicken breast (skinned and boned) was finely chopped and boiled for fifteen minutes with water (1 liter for every 1.5 kg of chicken). Residual chicken meat was removed by course filtration. The filtrate was flavored by the addition of carrot, onion, celery, salt, pepper, basil, parsley and tomato puree, and reboiled for a further fifteen minutes and then cooled before final filtration though fine muslin at 4° C. The yield from 1.5 kilograms of chicken and one liter of water was 870 mL of broth. A portion of the stock was assayed for the total beta-alanyl-dipeptide content (e.g., carnosine and anserine) and beta-alanine. Typical analyses were:

total beta-alanyl-dipeptides	74.5 mM
free beta-alanine	5.7 mM

The six male test subjects were of normal health and between 25-53 years of age, as shown in Table 4. The study commenced after an overnight fast (e.g., a minimum of 12 hours after the ingestion of the last meat containing meal). Subjects were given the option to consume a small quantity of warm water prior to the start of the study. Catheterization was begun at 08:30 and the study started at 09:00.

As a control, 8 milliliters per kilogram body weight of water was ingested (e.g., 600 mL in a subject weighing 75 kilograms).

In one session, 8 milliliters per kilogram body weight of broth containing approximately 40 milligrams per kilogram body weight of beta-alanine (e.g., in the form of anserine and carnosine) was ingested. For a subject weighing 75 kilograms, this amounted to the ingestion of 600 milliliters of broth containing 3 grams of beta-alanine. In another session, 3 milliliters per kilogram body weight of a liquid containing the test amount of beta-alanine with an additional 5 milliliters per kilogram body weight of water was ingested. In all sessions, subjects additionally consumed a further 8 milliliters per kilogram body weight of water (in 50 mL portions) during the period of 1 to 2 h after ingestion. A vegetarian pizza was provided after 6 hours. An ordinary diet was followed after 8 hours.

Plasma concentration curves following each treatment are depicted graphically in FIG **8**. Mean results of the administration of beta-alanine, broth, or carnosine according to the treatments schedule in Table 4. Plasma beta-alanine was below the limit of detection in all subjects on the control treatment. Neither carnosine or anserine were detected in plasma following ingestion of the chicken broth or any of the other treatments. Ingestion of the broth resulted in a peak concentration in plasma of 427.9 (SD 161.8) μM. Administration of carnosine equivalent to 20 milligrams per kilogram body weight of beta-alanine in one test subject resulted in an equivalent increase in the plasma beta-alanine concentration.

Administration of all treatments except control resulted in an increase in the plasma taurine concentration. The changes in taurine concentration mirrored closely those of beta-alanine. Administration of broth, a natural food, caused an equivalent increase in plasma taurine, indicating that the response occurs normally following the ingestion of most meals.

Example 3

The effect of administration of three doses of 10 milligrams per kilogram body weight of beta-alanine per day (i.e., administered in the morning, noon, and at night) for seven days on the plasma concentration profiles of beta-alanine and taurine were investigated. The plasma concentration profiles following administration of 10 milligrams per kilogram body weight of beta-alanine were studied in three subjects at the start and end of a seven-day period during which they were given three doses of the beta-alanine per day.

Three male subjects of normal health, aged between 33-53 years were studied. Test subjects received three doses per day of 10 milligrams per kilogram body weight of beta-alanine for eight days. In two subjects, this was followed by a further 7 days (days 9-15) when three doses of 20 milligrams per kilogram body weight per day were given. Subjects reported at 8 am to the blood collection laboratory on days 1 (prior to any treatment given), 8 and 15 following an overnight fast. Subjects were asked not to consume any meat containing meal during the 12 hours preceding the study. On each of these three test days subjects were catheterized and an initial blood sample taken when the beta-alanine was administered

at or close to 9 am, 12 noon, and 3 pm. Blood samples were drawn after 30, 60, 120 and 180 minutes, and analyzed for changes in the plasma concentration of beta-alanine and taurine. 24-hour urine samples were collected over each day of the study and analyzed by HPLC to determine the excretion of beta-alanine and taurine. The treatments are summarized in Table 5.

TABLE 5

Treatment Day beta-alanine	Day 1 10 mg/kg bwt	Day 8 10 mg/kg bwt	Day 15 20 mg/kg bwt
1	+	+	+
2	+	+	+
3	+	+	

The plasma beta-alanine concentrations are summarized in FIG. 9. Each dose resulted in a peak beta-alanine concentration at one-half hour or one hour after ingestion followed by a decline to a 0-10 micromolar basal level at three hours, just prior to administration of the next dose. The response on day 8 of the treatment tended to be less than on day 1, as indicated by the area under the plasma concentration curve.

Example 4

The effect of administration of three doses of 40 milligrams per kilogram body weight of beta-alanine per day (i.e., administered in the morning, noon, and at night) for 2 weeks on the carnosine content of muscle and isometric endurance at 66% of maximal voluntary contraction force was investigated.

Six normal male subjects, aged 25 to 32 years, that did not have evidence of metabolic or muscle disease were recruited into the study. The subjects were questioned regarding their recent dietary and supplementary habits. None of subjects was currently taking supplements containing creatine, or had done so in recent testing supplementation procedures. The physical characteristics of the test subjects are summarized in Table 6.

TABLE 6

Subject	Age (years)	Weight (kg)
1	29	78
2	31	94
3	29	105
4	25	65
5	31	81
6	25	75
7	53	76

Two days before treatment, a preliminary determination of maximal voluntary (isometric) contraction force (MVC) of knee extensors with the subject in the sitting position was carried out. MVC was determined using a Macflex system with subjects motivated by an instantaneous visual display of the force output. For each subject, two trials were carried out to determine endurance at 66% MVC sustained until the target force could no longer be maintained despite vocal encouragement. This first contraction was subsequently followed by a rest period of 60 seconds, with the subject remaining in the isometric chair. After the rest period, a second contraction was sustained to fatigue. Following a second rest of 60 seconds, a third contraction to fatigue was undertaken.

One day before treatment, the subjects reported to the isometric test laboratory between 8 and 10 am. MVC was determined and endurance at 66% MVC over three contractions with 60 second rest intervals, as described above, was determined. Measurements were determined using the subject's dominant leg. A biopsy of the lateral portion of the vastus lateralis was taken again from the dominant leg.

On day 1 of the treatment study, subjects reported to the blood sampling laboratory at 8 am following an overnight fast and a minimum of 12 hours since the last meat containing meal. Following catheterization and a basal blood sample, each subject followed the supplementation and blood sampling protocol described in Example 3. A dose of 10 milligrams per kilogram body weight of beta-alanine was administered at time 0 (9 am), 3 hours, and 6 hours.

On days 2-15, subjects continued to take three doses of 10 milligrams per kilogram body weight of beta-alanine.

In the morning of day 14, post-treatment isometric exercise tests were conducted on the dominant leg to determine MVC and endurance at 66% MVC relative to the 66% MVC measured on the day prior to treatment. In the afternoon, a muscle biopsy was taken of the vastus lateralis from close to the site of the biopsy taken on the day before treatment.

On day 15, the procedures followed on day 1 were repeated to determine any overall shift in the plasma concentration profile of beta-alanine and taurine over the 15 days of supplementation. Mean changes in plasma beta-alanine over 9 hours following the oral ingestion of 10 milligrams per kilogram body weight of beta-alanine at 0, 3 and 6 hours on days 1 and 15 when dosing at 3×10 milligrams per kilogram body weight per day are shown in FIG. 10.

One additional test subject (number 7) followed the study, taking three doses 10 milligrams per kilogram body weight for 7 days followed by three doses of 20 milligrams per kilogram body weight for 7 days. No muscle biopsies were taken from this test subject.

There was no apparent change in the muscle carnosine content in the muscle of the six subjects biopsied. Changes in plasma taurine concentrations in the six subjects mirrored those of beta-alanine, as noted in Example 2.

Values from the MVC and endurance at 66% MVC measurements one day before treatment and after 14 days after treatment with three doses of 10 milligrams per kilogram body weight of beta-alanine are listed in Table 7. The mean endurance time at 66% MVC increased in 5 of the 6 subjects. An increase was also seen in subject 7 who had taken the higher dose.

TABLE 7

Subject	MVC 1st try N	MVC 2nd try N	time @ 66% MVC 1st seconds	time @ 66% MVC 2nd seconds	time @ 66% MVC 3rd seconds	Total Contraction Time seconds
Pre						
1	784.5	821.9	48.53	29.03	23.78	100.83
2	814.4	886.2	48.40	26.03	16.90	91.33
3	984.9	970.4	38.15	26.03	16.78	80.95
4	714.6	740.4	89.03	56.15	45.65	190.83
5	1204.8	1217.2	37.65	27.64	21.53	86.83
6	722.4	716.8	46.78	29.40	21.90	98.08
Pre mean	870.9	892.1	51.4	32.4	24.3	108.1
Pre SD	190.6	184.6	19.1	11.7	10.8	41.2
Post						
1	895.6	908.0	47.08	30.38	24.03	101.48
2	832.2	908.0	46.65	31.28	18.40	96.33
3	973.7	952.2	42.65	25.03	16.03	83.70
4	814.1	863.9	114.40	64.28	48.53	227.20

TABLE 7-continued

Subject	MVC 1st try N	MVC 2nd try N	time @ 66% MVC 1st seconds	time @ 66% MVC 2nd seconds	time @ 66% MVC 3rd seconds	Total Contraction Time seconds
5	1246.6	1233.0	42.03	22.78	19.40	84.20
6	760.8	773.3	52.28	31.53	25.95	109.73
Post mean	920.5	939.7	57.5	34.2	25.4	117.1
Post SD	175.7	156.0	28.1	15.2	11.9	54.9
Subject 7						
Pre	858.18	861.54	54.0			
Post	792.54	851.41	62.0			

Example 5

The effect of 4 weeks of beta-alanine supplementation using two dosing regimens and an isomolar dose of L-carnosine (beta-alanylhistidine) administered over 4 weeks on the muscle carnosine content were investigated.

Fifteen male subjects, aged 20 to 29 years with no obvious signs of clinical disease and with heights and weights within the normal range, were recruited into the study (Table 8). All subjects participated in one or more sports and all ate a mixed diet containing variable amounts of meat. A record of each subject's approximate intake of meat during the course of the investigation was made.

TABLE 8

Summary of subjects' physical characteristics for each of the three treatment groups:

Treatment	AGE Mean ± SD	HEIGHT Mean ± SD	MASS Mean ± SD
1	24.4 ± 2.7	182.3 ± 7.5	80.0 ± 15.9
2	23.8 ± 1.9	180.9 ± 5.4	80.6 ± 8.6
3	24.0 ± 3.8	180.1 ± 3.8	80.4 ± 12.1

Five subjects were allocated to one of three treatment groups (1, 2, and 3). During the study, their diet was supplemented with either beta-alanine or carnosine as described in Table 9 (FIG. 17). The supplements were provided in soft gelatine capsules containing either 400 mg beta alanine or 500 mg carnosine.

In Group 1, beta-alanine was administered in 4 separate doses throughout the day (qid) at a steady rate for four weeks.

In Group 2, beta-alanine was administered as 8 separate doses throughout the day, rather than as 4 doses, in an attempt to maintain a more even increase in the blood-plasma concentration. In addition, the dose was increased progressively each week by 800 mg per day.

In Group 3, carnosine was administered at approximately the same isomolar dose as in Group 2, again divided into 8 doses. This treatment, therefore, contained approximately the same amount of beta-alanine as in Group 2, when hydrolyzed to its constituent amino acids.

The subjects took the supplements at the times indicated in Table 9 (FIG. 17). A single muscle biopsy of the vastus lateralis was taken before and at the end of the supplementation using the percutaneous needle biopsy procedure of Bergstrom (1962). In brief, the procedure involves the insertion of a hollow bored needle under local anesthetic and sterile conditions to obtain specimens around 20-40 mg containing approximately 100-700 muscle fibers. The skin and subcutaneous tissue is anesthetized with 1% lignocaine (avoiding contact with the muscle). An incision is made to the skin and deep fascia with a scalpel blade. The needle minus central rod is inserted into the muscle. The muscle bulk is pressed into a needle side-window. A sample is cut by ramming an inner, sharpened cylinder along the needle. The needle is removed and the central rod is used to evacuate the specimen. The wound is then closed.

Muscle samples from the subjects were frozen in liquid nitrogen, freeze-dried and analyzed for muscle carnosine and taurine contents by HPLC. Table 9 (FIG. 17) shows a breakdown of the dosing strategies employed in each of the three treatment groups. Results: There was no change in body mass in either beta-alanine or carnosine supplemented subjects.

Changes in Muscle Carnosine (Table 10 and FIGS. **12** and **13**).

A significant increase in muscle carnosine content was recorded for the subjects in Groups 1 and 3. In Group 2, one subject (no. 10) with the highest initial carnosine content (initial carnosine content: 33.3 mmol/kg dry muscle) showed no change in his muscle carnosine content (post content: 33.7 mmol/kg dm). When this subject was deleted from Group 2, this group showed a significant increase of the same order as seen in the other Groups. Subject 10 was a medium to high consumer of meat and otherwise unremarkable.

Supplementation with either beta-alanine or carnosine at the same dose (Groups 2 and 3) appeared to be equally effective in increasing the muscle carnosine content.

The pattern of change is reminiscent of the changes observed with creatine loading and may suggest that there is a threshold which is quickly reached, with further supplementation having no further effect. In the case of subject 10, while not wishing to be bound by this theory, a threshold appears to have been reached even before the start of supplementation. However, there are exceptions to the notion of an upper threshold, notably subjects 6 (post supplementation carnosine: 45.9 mmol/kg dry muscle) and 15 (post supplementation carnosine: 68.9 mmol/kg dm). Subjects 6 and 15 were unremarkable in either their dietary patterns or participation in physical exercise.

Table 10 is a summary of data for carnosine muscle concentrations for treatment Groups 1 to 3. Treatment Group 2, in italics, is without subject 10 who did not exhibit an increase in muscle carnosine concentration. The initial carnosine concentration in subject 10 was the highest of all subjects and may have already been at an "upper threshold" level prior to supplementation.

TABLE 10

	Treatment			
	1 n = 5	2 n = 5	2 n = 4	3 n = 5
Mean pre	19.58	24.23	21.96	23.15
SD pre	3.71	5.27	1.64	5.07
Mean post	27.38	35.27	35.67	39.52
SD post	2.96	6.18	7.08	16.95
Mean difference	7.80	11.04	13.72	16.37
SD difference	0.81	9.20	8.08	12.06
Sign	***	ns	*	*
Min difference	6.99	0.35	8.62	7.05
Max difference	9.08	25.77	25.77	37.39
Mean % change	42.1	51.6	64.2	65.8
SD % change	14.9	46.5	42.7	31.8
Min % change	31.5	1.1	41.0	38.2
Max % change	68.0	128.2	128.2	118.7

Changes in Muscle beta-Alanine and Histidine (Table 11 and FIG. **14**)

The muscle beta-alanine concentration was below the limit of detection (<0.2 mmol/kg dm) before and at the end of supplementation. In some subjects, a final dose of beta-alanine was taken within 1 to 2 hours of the final muscle biopsy.

There was no change in the muscle histidine concentration with supplementation with either beta-alanine or carnosine, the latter having the potential to release histidine into the general circulation. There was no decrease in the histidine concentration in response to the increased synthesis of carnosine (each mole requiring one mole of histidine).

Changes in Muscle Taurine (Table 12 and FIG. **15** and FIG. **16**)

While beta-alanine at high concentrations may interfere with the uptake of taurine into tissues, previous observations show an increase in the plasma taurine concentration and loss of taurine in urine following both beta-alanine and carnosine administration, no loss of muscle taurine was noted in this study in any of the three Groups. Marked changes in the muscle taurine content occurred in some individuals, but both increases and decreases were observed. FIG. **15** is a graph illustrating data showing muscle concentration (mean+SD) of taurine before and post supplementation in four different treatment groups. FIG. **16** is a graph illustrating data showing muscle concentration (mean+SD) of taurine before and post supplementation in different subjects.

Conclusions

These studies demonstrate that the supplements of beta-alanine and carnosine of the invention have the potential to increase the muscle carnosine content. Based on the test results, they appear to be equally effective in increasing carnosine in tissue.

The changes in the muscle buffering capacity help maintain the intracellular microenvironment during intense exercise, countering the accumulation of H^+. As such, supplementation with beta-alanine or compounds delivering beta-alanine on ingestion may have a positive effect on exercise capacity in sports and those general daily activities leading to lactate accumulation. In view of the other chemical activities ascribed to carnosine (as an anti-oxidant and anti-glycating agent), an increase in carnosine concentration may have other beneficial effects apart from those arising from an increase in muscle buffering capacity.

Four weeks of supplementation did not result in any apparent loss of taurine in the muscles.

TABLE 11

summary data for histidine muscle concentrations in treatment Groups 1 to 3:

	Treatment		
	1 n = 5	2 n = 5	3 n = 5
mean pre	5.76	5.56	7.01
SD pre	0.59	0.63	2.60
mean post	5.12	5.51	5.38
SD post	1.17	0.87	1.39
mean diff	−0.64	−0.05	−1.63
SD diff	1.24	0.70	2.87
Sign	ns	ns	ns
% change	−10.59	−0.78	−17.47
SD % change	21.65	12.65	29.56

TABLE 12

Summary data for taurine muscle concentrations in treatments Groups 1 to 3:

	Treatment		
	1 n = 5	2 n = 5	3 n = 5
Mean pre	36.52	28.68	35.40
SD pre	7.77	5.29	8.92
Mean post	33.70	27.54	32.32
SD post	7.98	8.77	15.19
Mean difference	−2.82	−1.15	−3.08
SD difference	7.96	6.04	13.61
Sign	ns	ns	ns
% change	−6.28	−4.46	−7.66
SD % change	21.54	24.06	34.97

A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.

What is claimed is:

1. A composition, comprising:

glycine; and

a) an amino acid selected from the group consisting of a beta-alanine, an ester of a beta-alanine, and an amide of a beta-alanine, or

b) a di-peptide selected from the group consisting of a beta-alanine di-peptide and a beta-alanylhistidine di-peptide.

2. The composition of claim **1**, wherein the beta-alanylhistidine dipeptide is a carnosine, an anserine or a balenine.

3. The composition of claim **1**, further comprising at least creatine or a carbohydrate.

4. The composition of claim **1**, wherein the composition is a pharmaceutical composition.

5. The composition of claim **1**, wherein the composition is a dietary supplement or a sports drink.

6. The composition of claim **5**, wherein the dietary supplement or sports drink is a supplement for humans.

7. A composition comprising at least 0.2, 0.3, 0.4, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 or 5 grams of a peptide or an ester comprising a beta-alanine per dosage.

8. A composition comprising at least 0.2, 0.3, 0.4, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 grams of a peptide or an ester comprising a beta-alanine in an injectable form per dosage.

9. The composition of claim **7** or claim **8**, wherein the peptide comprises a beta-alanylhistidine dipeptide.

10. The composition of claim **9**, wherein the beta-alanylhistidine dipeptide comprises a carnosine, an anserine or a balenine.

11. A composition for humans comprising at least 200, 250, 300, 450, 500, 550, 600, 650, 700, 750 or 800 mg of a beta-alanine per dosage.

12. The composition of claim **11**, wherein the composition is formulated in an ingestible or an injectable formulation.

13. The composition of claim **12**, wherein the ingestible formulation is a drink, a gel, a food or a tablet.

14. The composition of claim **11**, wherein the peptide comprises a beta-alanylhistidine dipeptide.

15. The composition of claim **13**, wherein the beta-alanylhistidine dipeptide comprises a carnosine, an anserine or a balenine.

16. The composition of claim **1**, wherein the composition is formulated for oral, enteral or parenteral administration.

17. The composition of claim **1**, wherein the composition is formulated for infusion through the skin of a subject.

18. The composition of claim **17**, wherein the composition is a topical cream or a patch.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 7,504,376 B2
APPLICATION NO. : 10/717217
DATED : March 17, 2009
INVENTOR(S) : Harris et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the Title page,

[*] Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 USC 154(b) by 493 days.

Delete the phrase "by 493 days" and insert -- by 979 days --

Signed and Sealed this

Twenty-fourth Day of August, 2010

David J. Kappos

David J. Kappos
Director of the United States Patent and Trademark Office

EXHIBIT G

US007825084B2

(12) **United States Patent**
Harris et al.

(10) **Patent No.:** **US 7,825,084 B2**
(45) **Date of Patent:** ***Nov. 2, 2010**

(54) **METHODS AND COMPOSITIONS FOR INCREASING THE ANAEROBIC WORKING CAPACITY IN TISSUES**

(75) Inventors: **Roger Harris**, Newmarket (GB); **Mark Dunnett**, Tuddenham (GB)

(73) Assignee: **Natural Alternatives International, Inc.**, San Diego, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

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(60) Continuation of application No. 10/717,217, filed on Nov. 18, 2003, now Pat. No. 7,504,376, and a continuation-in-part of application No. 10/209,169, filed on Jul. 30, 2002, now Pat. No. 6,680,294, which is a continuation of application No. 09/757,782, filed on Jan. 9, 2001, now Pat. No. 6,426,361, which is a continuation of application No. 09/318,530, filed on May 25, 1999, now Pat. No. 6,172,098, which is a division of application No. 08/909,513, filed on Aug. 12, 1997, now Pat. No. 5,965,596.

(60) Provisional application No. 60/462,238, filed on Apr. 10, 2003.

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(58) **Field of Classification Search** 514/3, 514/23, 385, 400, 561

See application file for complete search history.

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(57) **ABSTRACT**

Provided are compositions comprising beta-alanylhistidine peptides and beta-alanines, and methods for administering these peptides and amino acids. In one aspect, the compositions and methods cause an increase in the blood plasma concentrations of beta-alanine and/or creatine.

18 Claims, 19 Drawing Sheets

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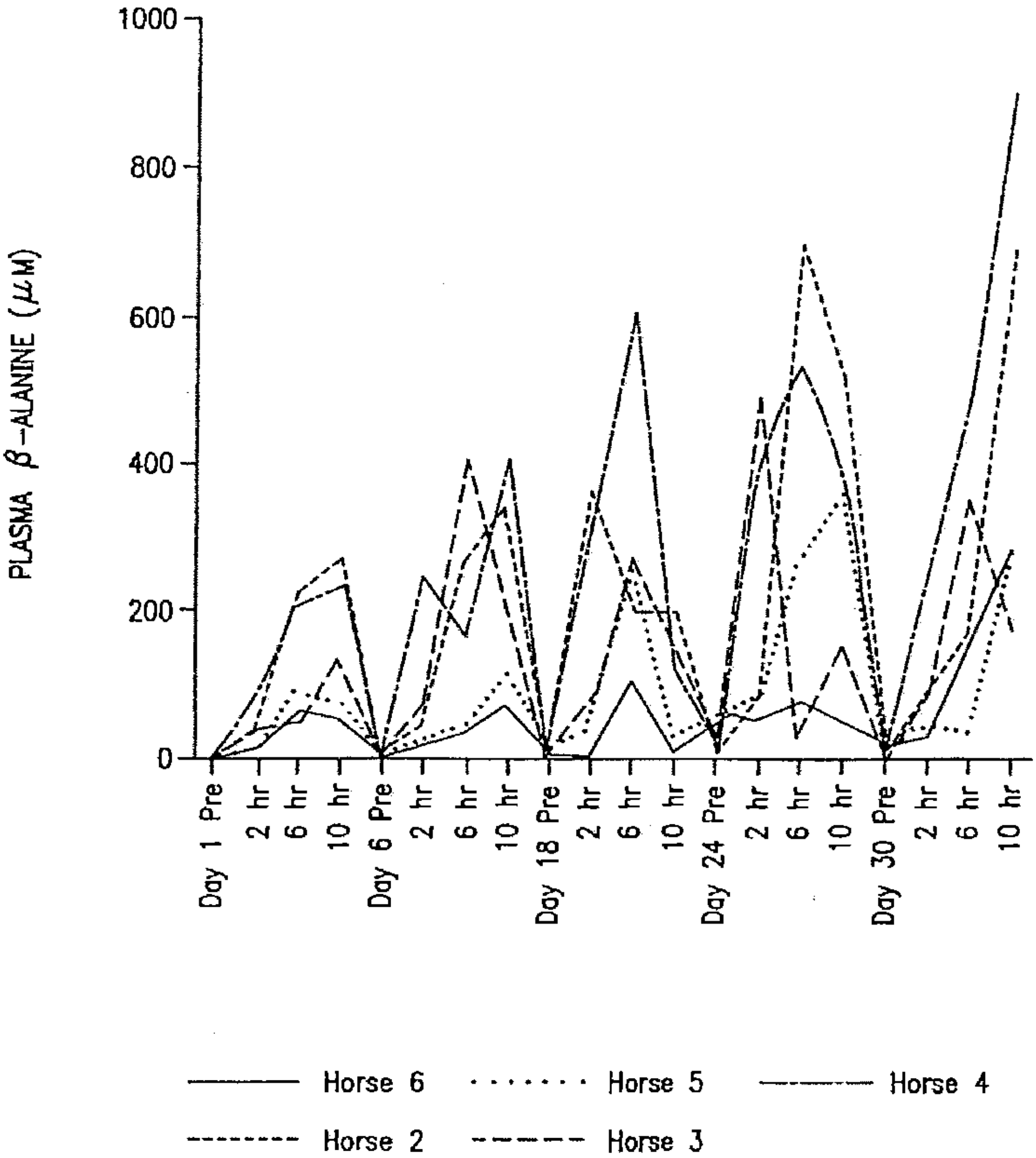

1000
800
600
400
200
0
PLASMA β-ALANINE (μM)
Day 1 Pre
2 hr
6 hr
10 hr
Day 6 Pre
2 hr
6 hr
10 hr
Day 18 Pre
2 hr
6 hr
10 hr
Day 24 Pre
2 hr
6 hr
10 hr
Day 30 Pre
2 hr
6 hr
10 hr
Horse 6
Horse 5
Horse 4
Horse 2
Horse 3

FIG. 1

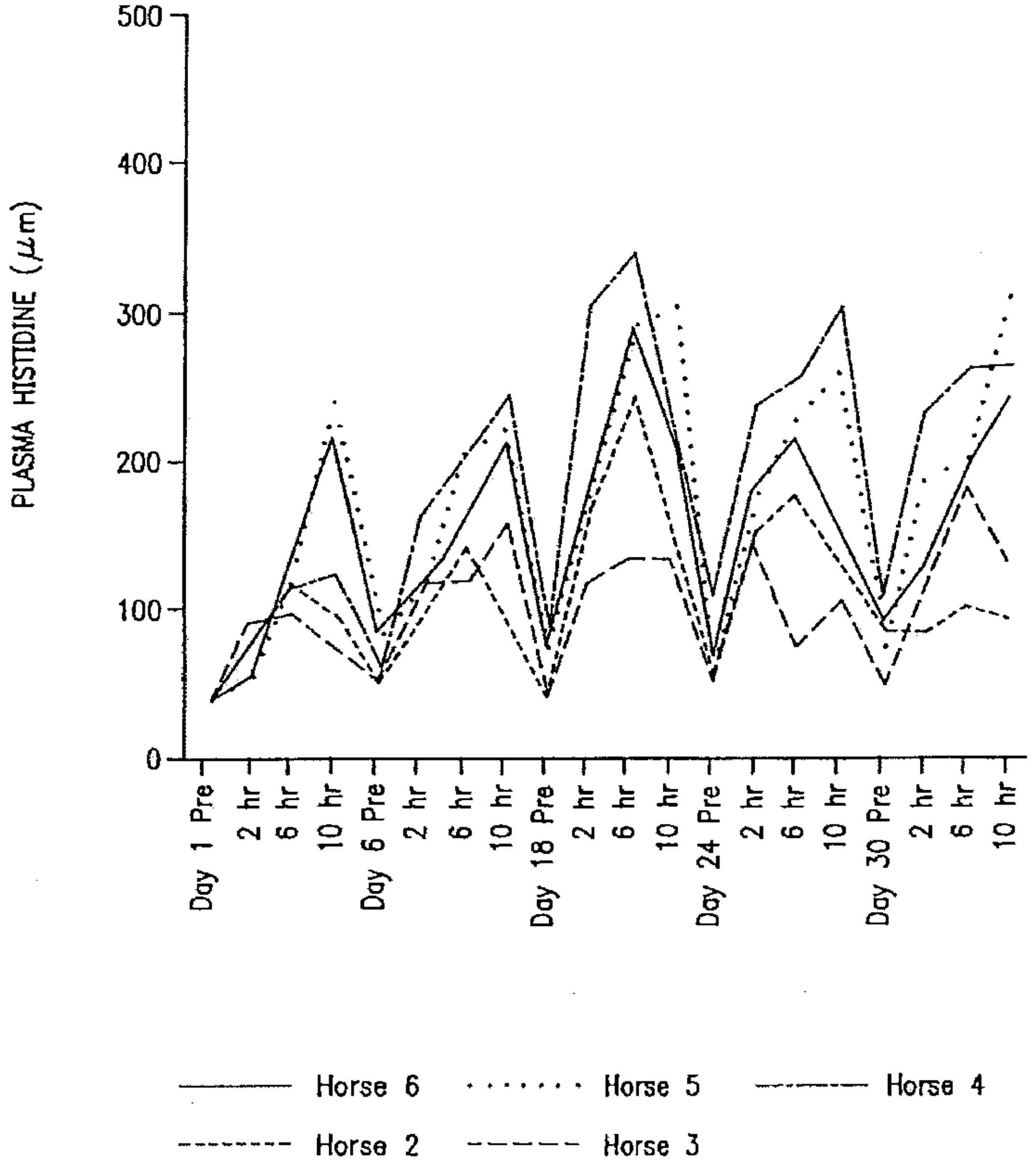

PLASMA HISTIDINE (μm)
500
400
300
200
100
0
Day 1 Pre
2 hr
6 hr
10 hr
Day 6 Pre
2 hr
6 hr
10 hr
Day 18 Pre
2 hr
6 hr
10 hr
Day 24 Pre
2 hr
6 hr
10 hr
Day 30 Pre
2 hr
6 hr
10 hr
Horse 6
Horse 5
Horse 4
Horse 2
Horse 3

FIG. 2

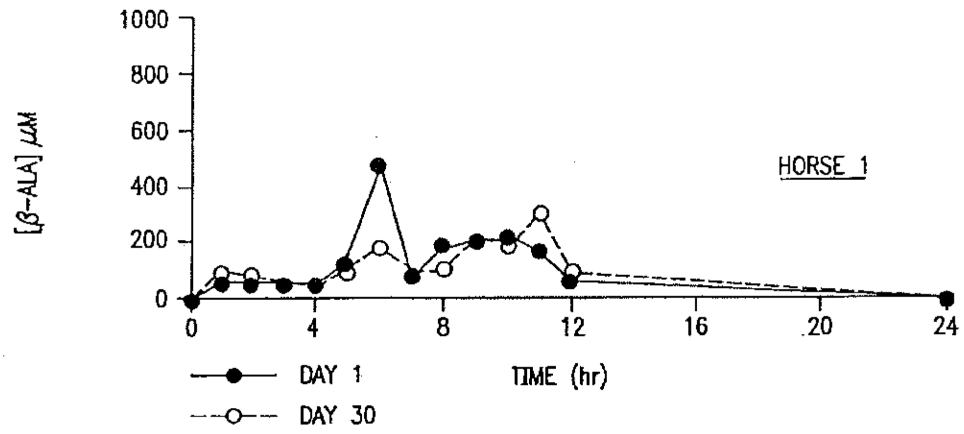


FIG. 3A

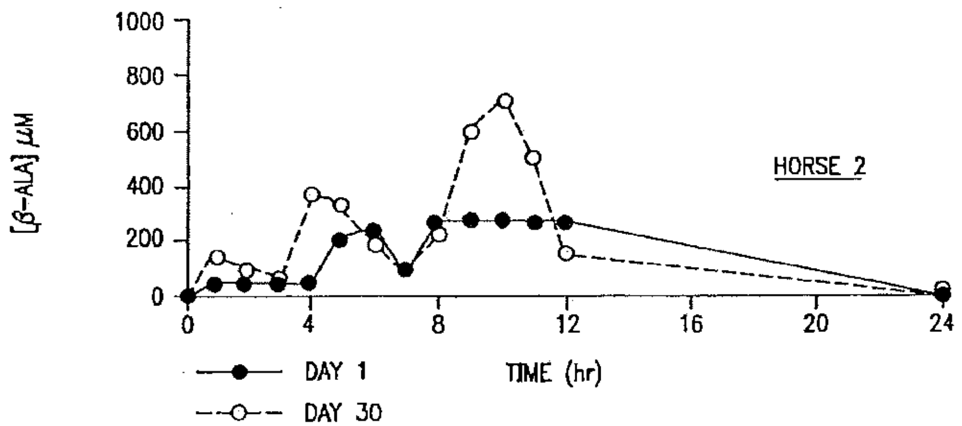


FIG. 3B

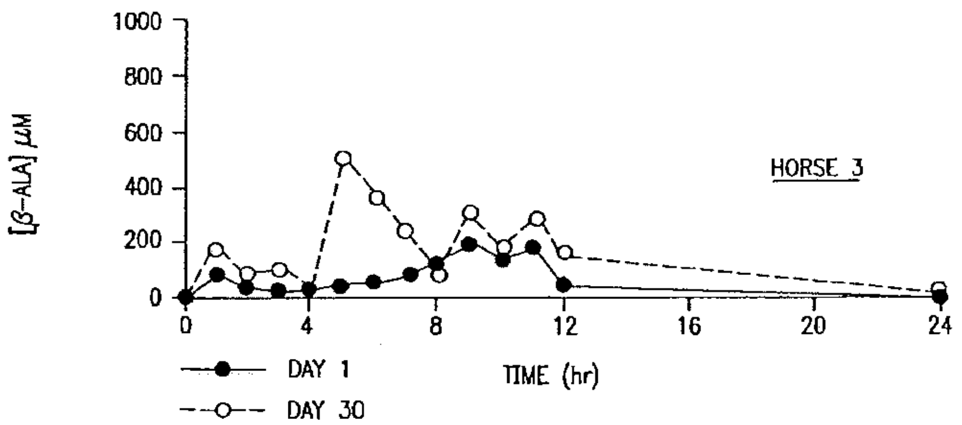


FIG. 3C

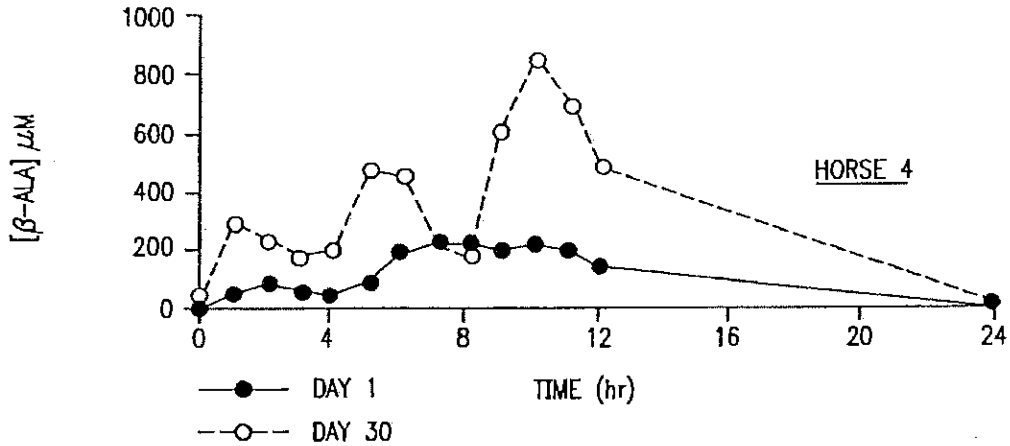


FIG. 3D

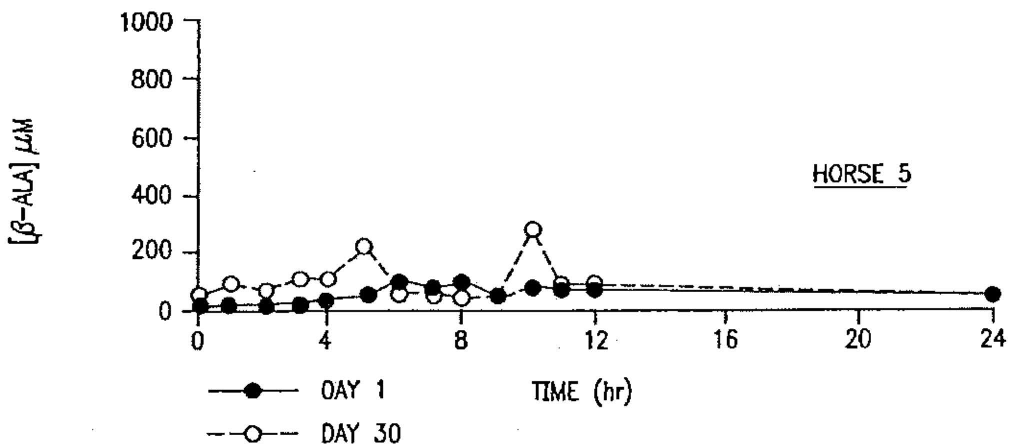


FIG. 3E

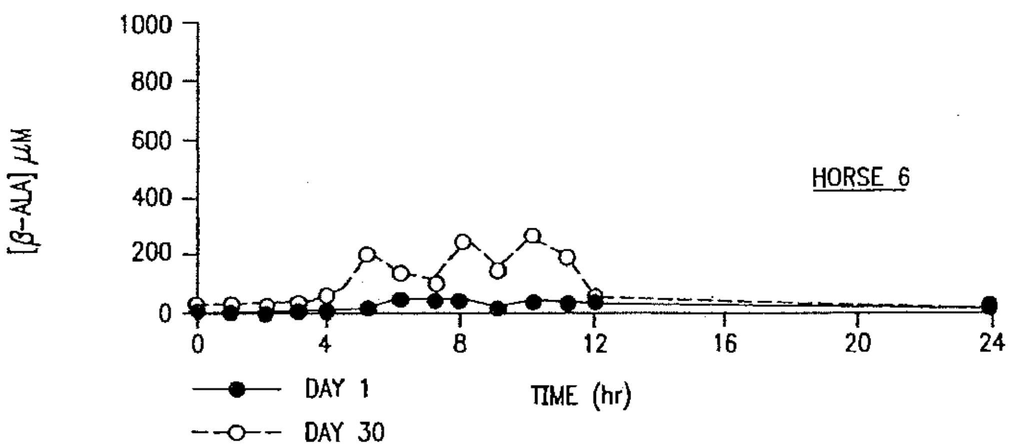


FIG. 3F

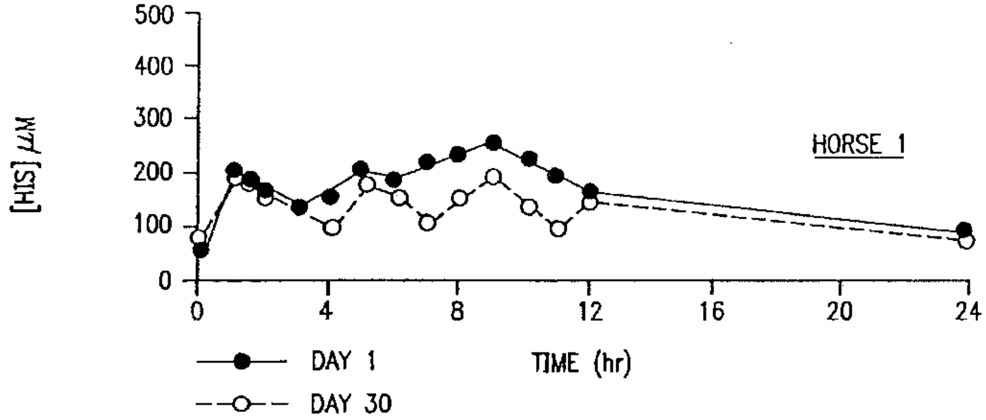


FIG. 4A

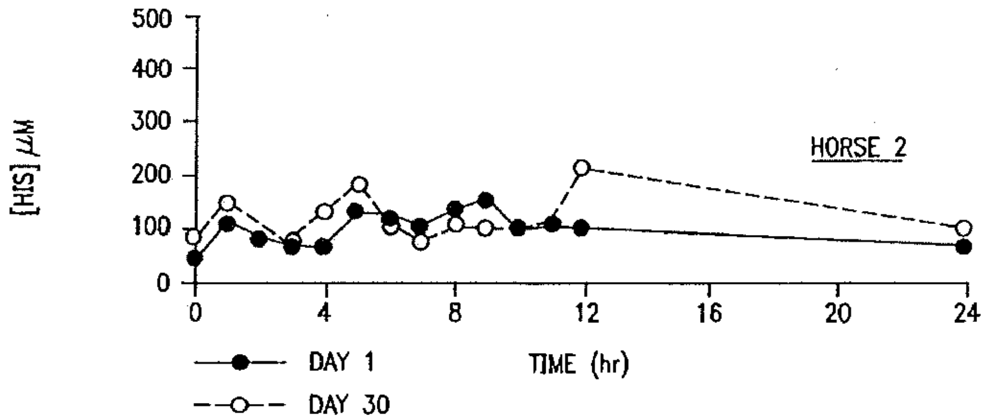


FIG. 4B

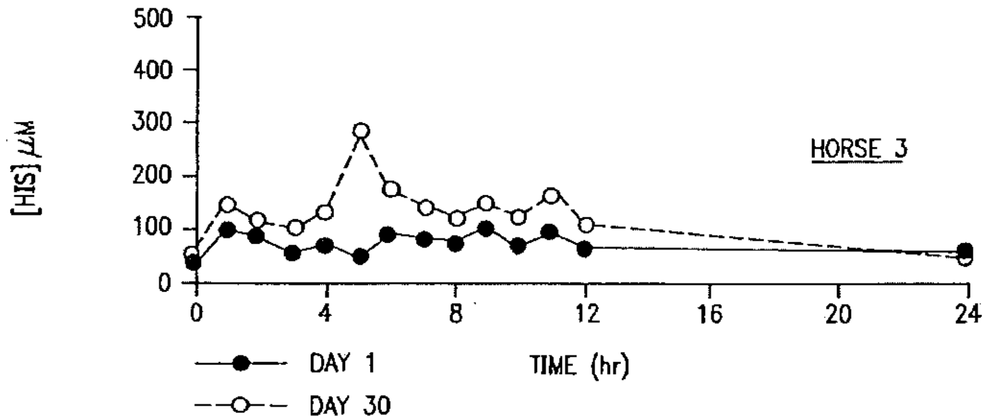


FIG. 4C

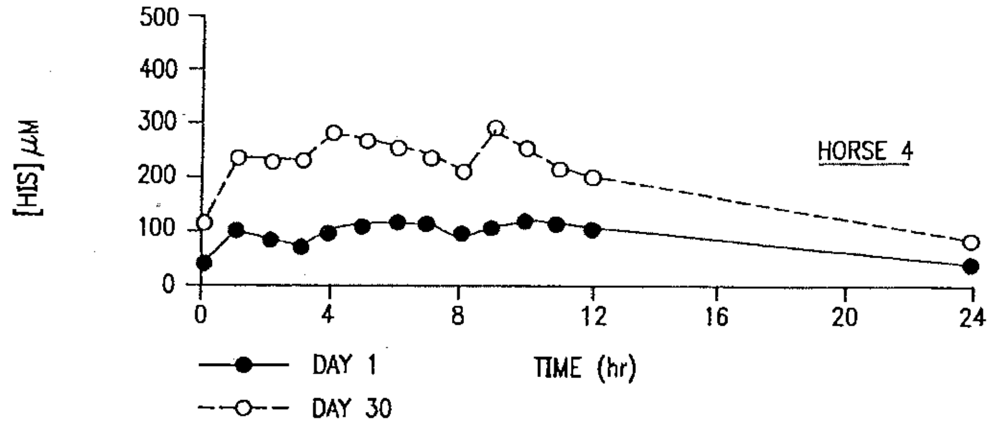


FIG. 4D

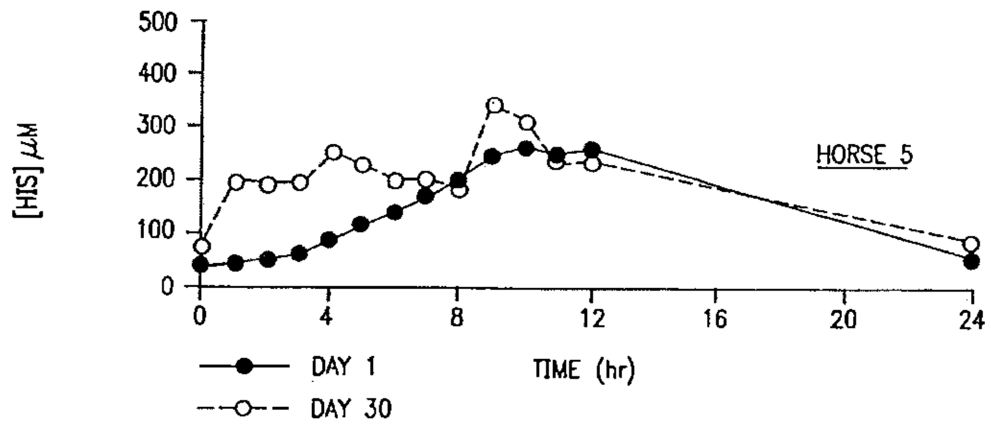


FIG. 4E

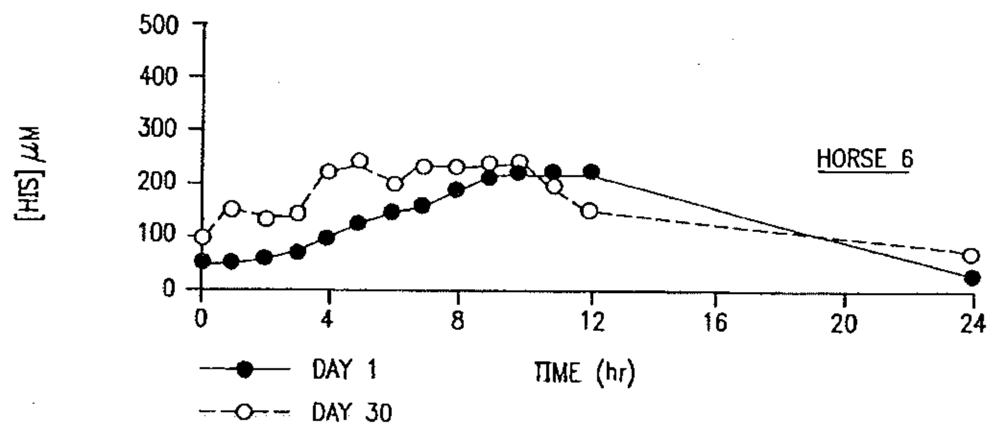


FIG. 4F

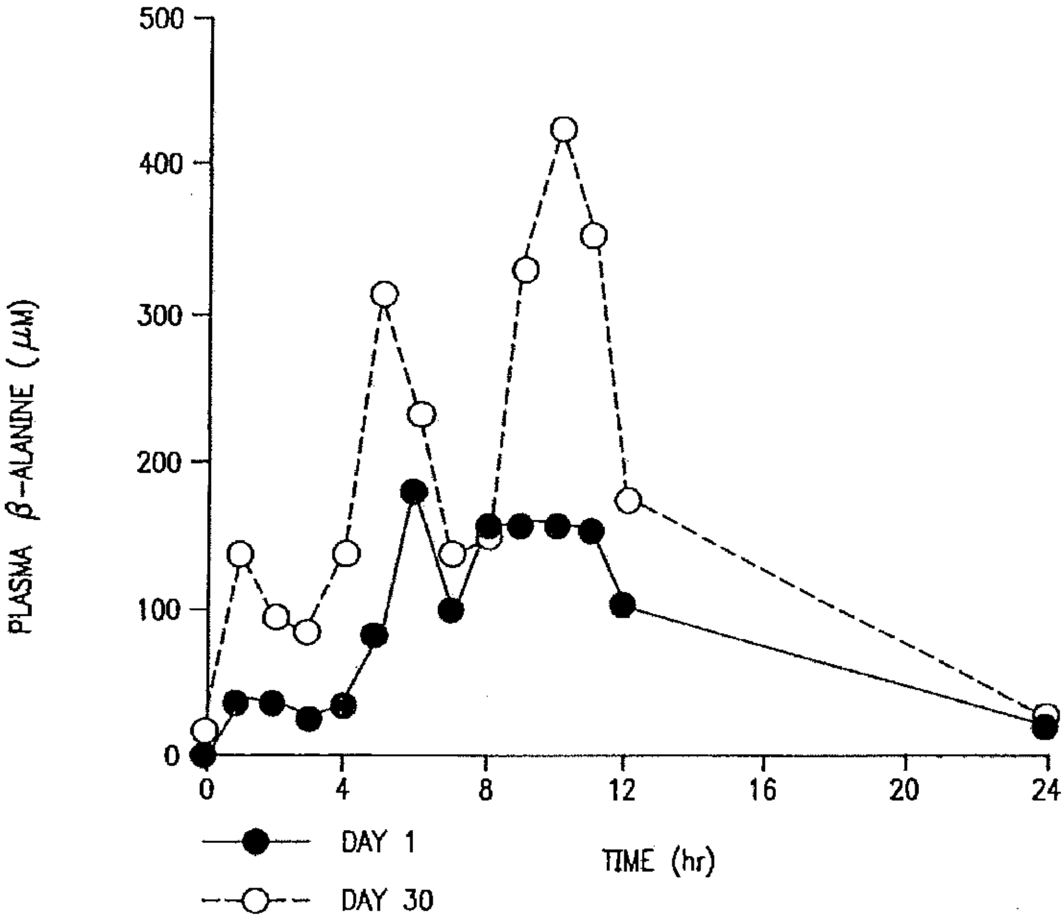

500
400
300
200
100
0
PLASMA β-ALANINE (μM)
0
4
8
12
16
20
24
TIME (hr)
DAY 1
DAY 30

FIG. 5

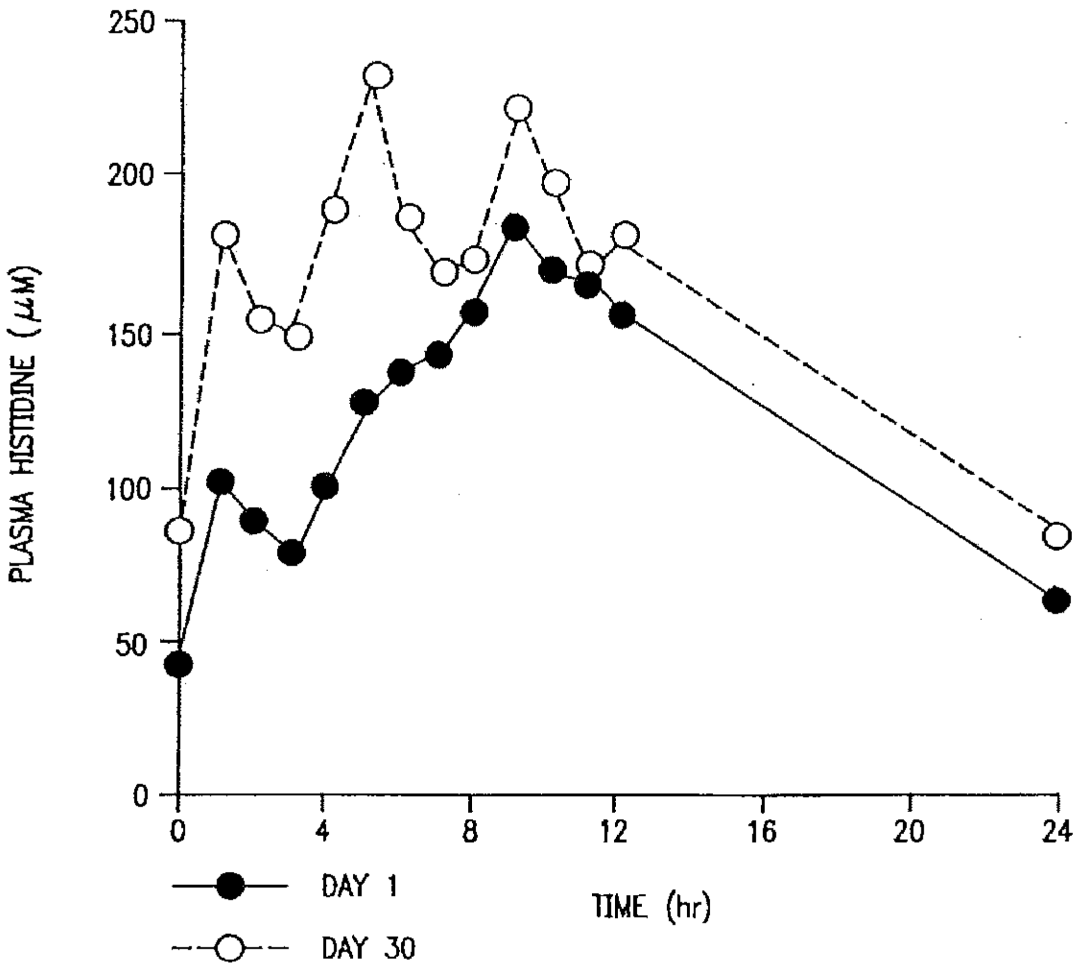

250
200
150
100
50
0
PLASMA HISTIDINE (μM)
0
4
8
12
16
20
24
TIME (hr)
DAY 1
DAY 30

FIG. 6

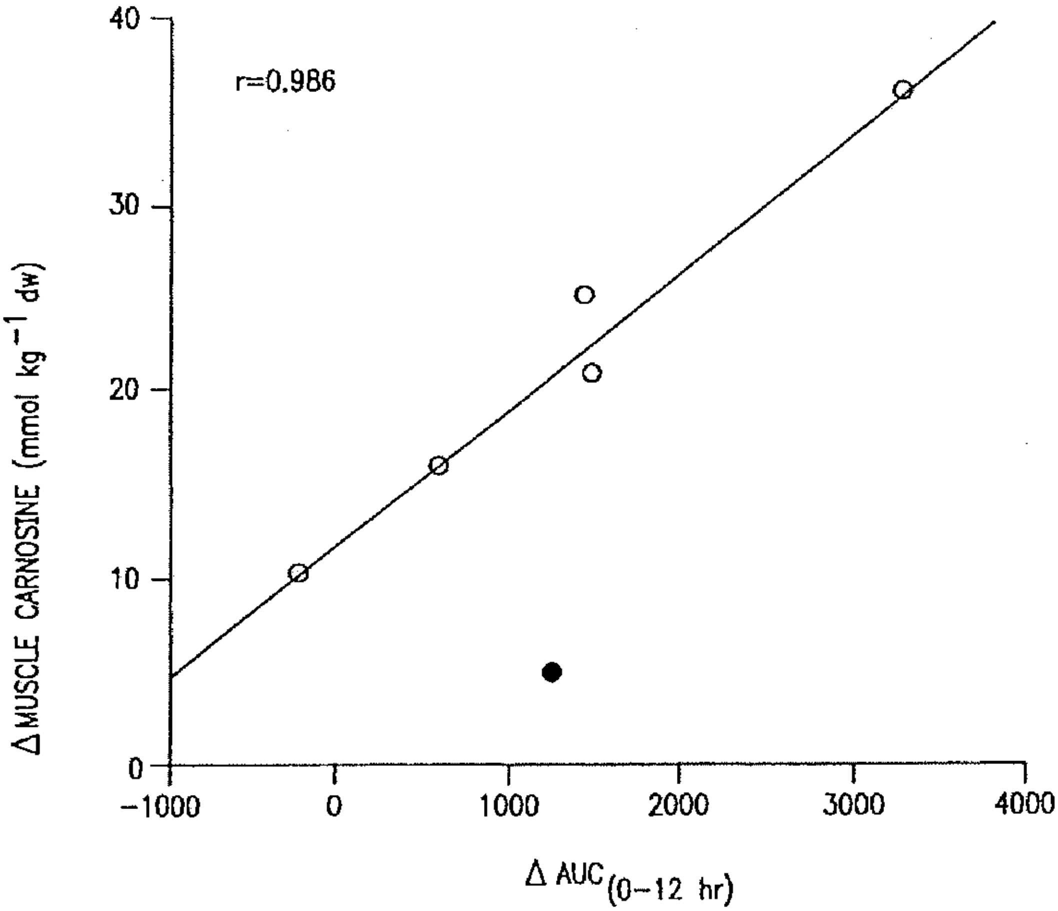
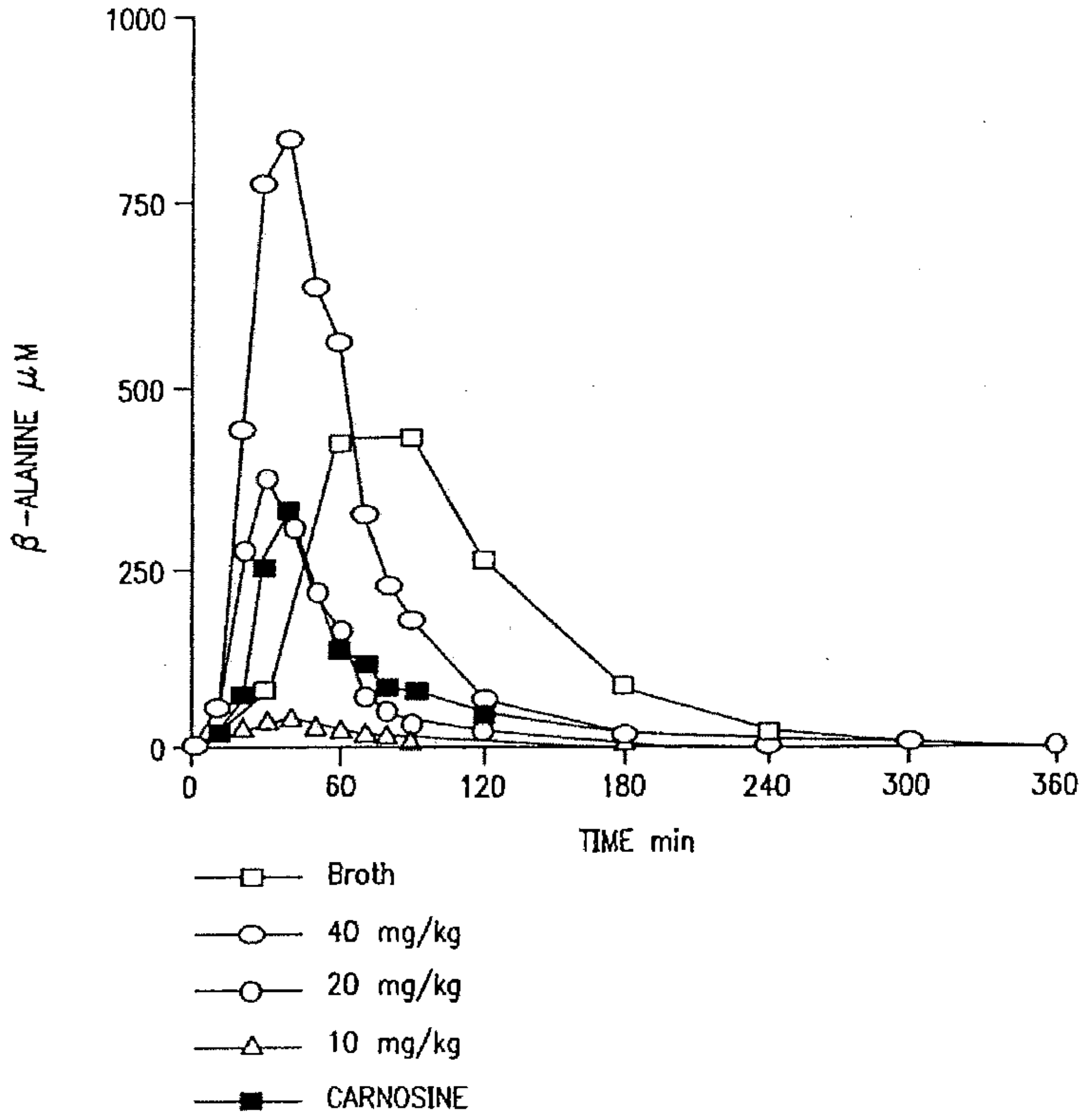

40
30
20
10
0
r=0.986
Δ MUSCLE CARNOSINE (mmol kg⁻¹ dw)
−1000
0
1000
2000
3000
4000
Δ AUC (0−12 hr)

FIG. 7



1000
750
500
250
0
β-ALANINE μM
0
60
120
180
240
300
360
TIME min
Broth
40 mg/kg
20 mg/kg
10 mg/kg
CARNOSINE

FIG. 8

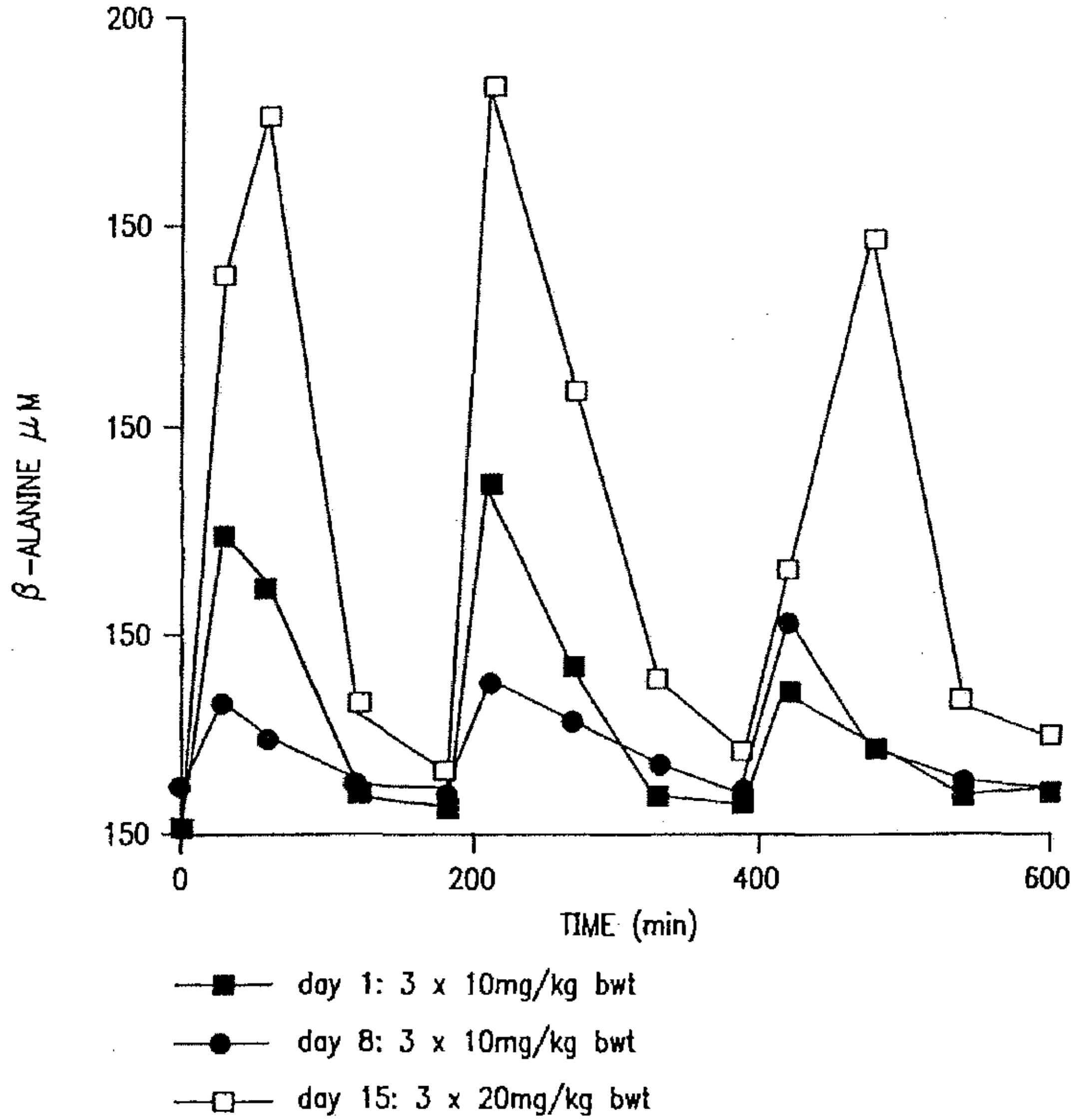

200
150
150
150
150
β-ALANINE μM
0
200
400
600
TIME (min)
day 1: 3 x 10mg/kg bwt
day 8: 3 x 10mg/kg bwt
day 15: 3 x 20mg/kg bwt

FIG. 9

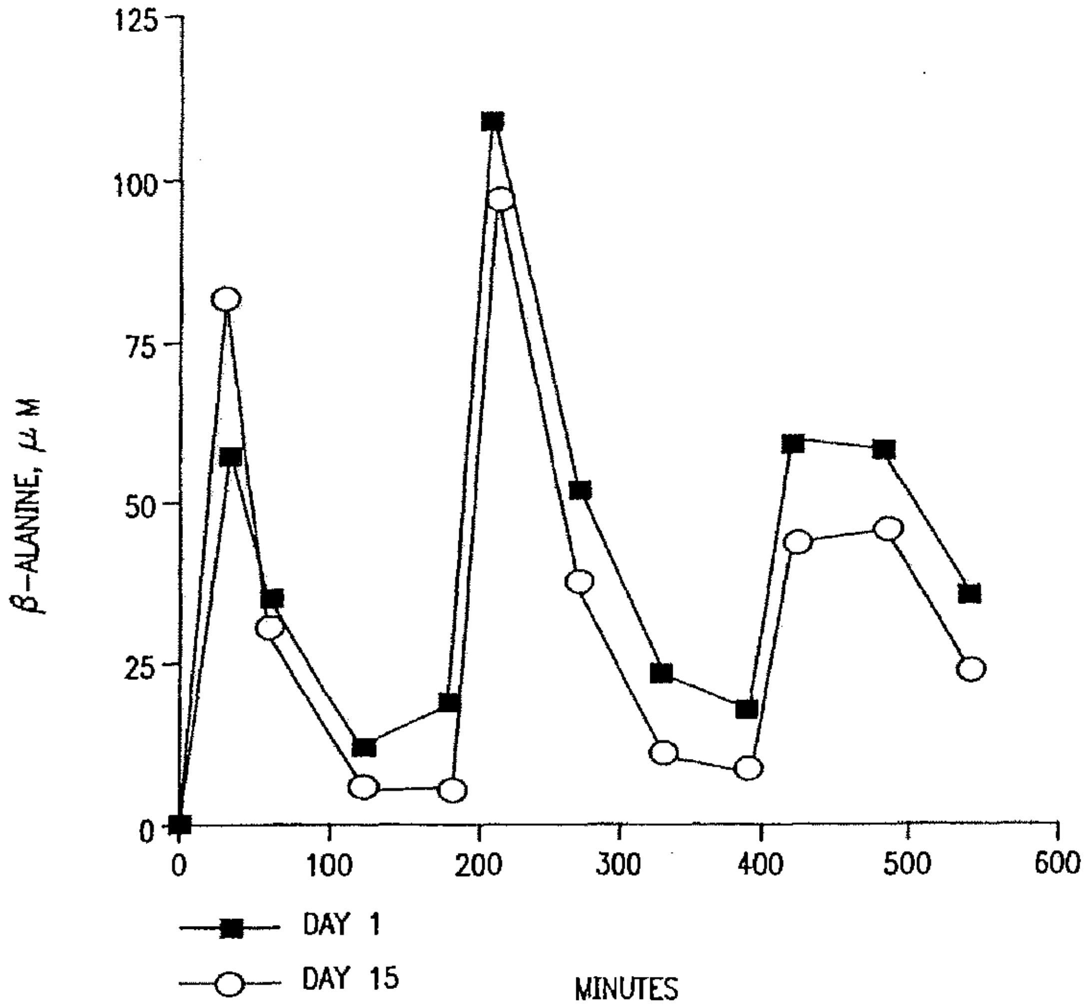

125
100
75
50
25
0
β–ALANINE, μ M
0
100
200
300
400
500
600
MINUTES
DAY 1
DAY 15

FIG. 10

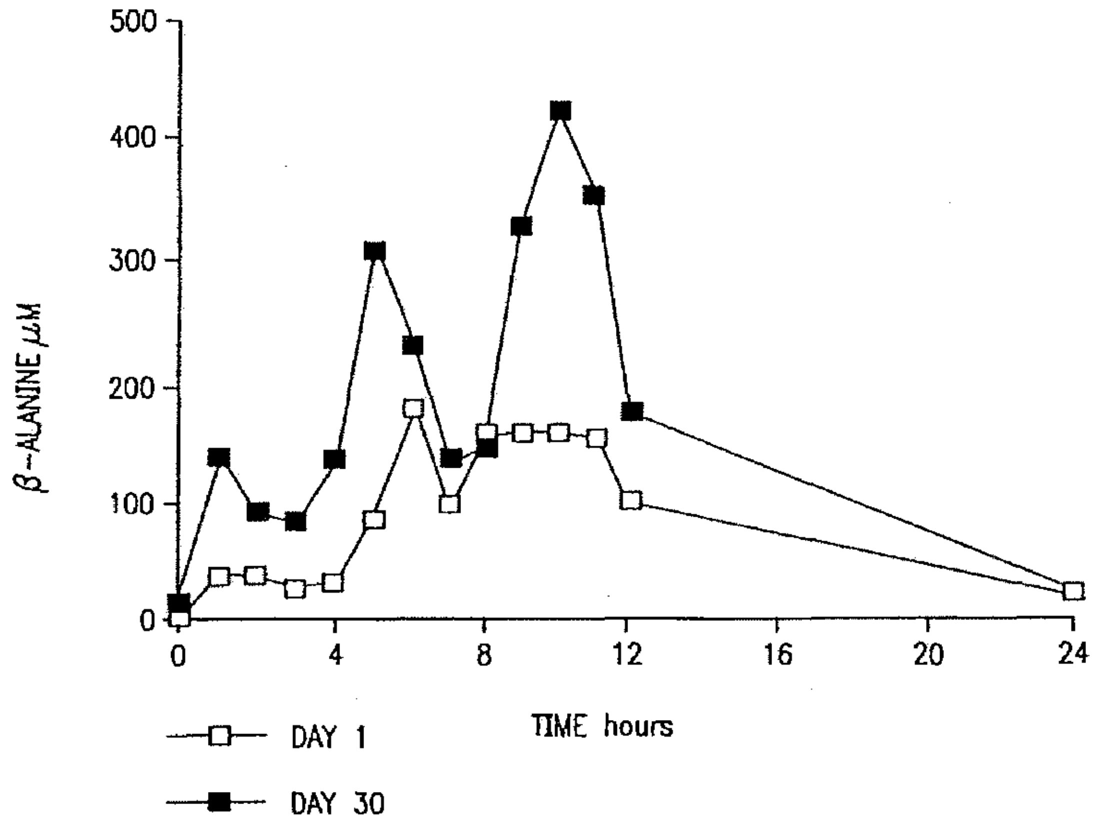

500
400
300
200
100
0
β-ALANINE μM
0
4
8
12
16
20
24
TIME hours
DAY 1
DAY 30

FIG. 11

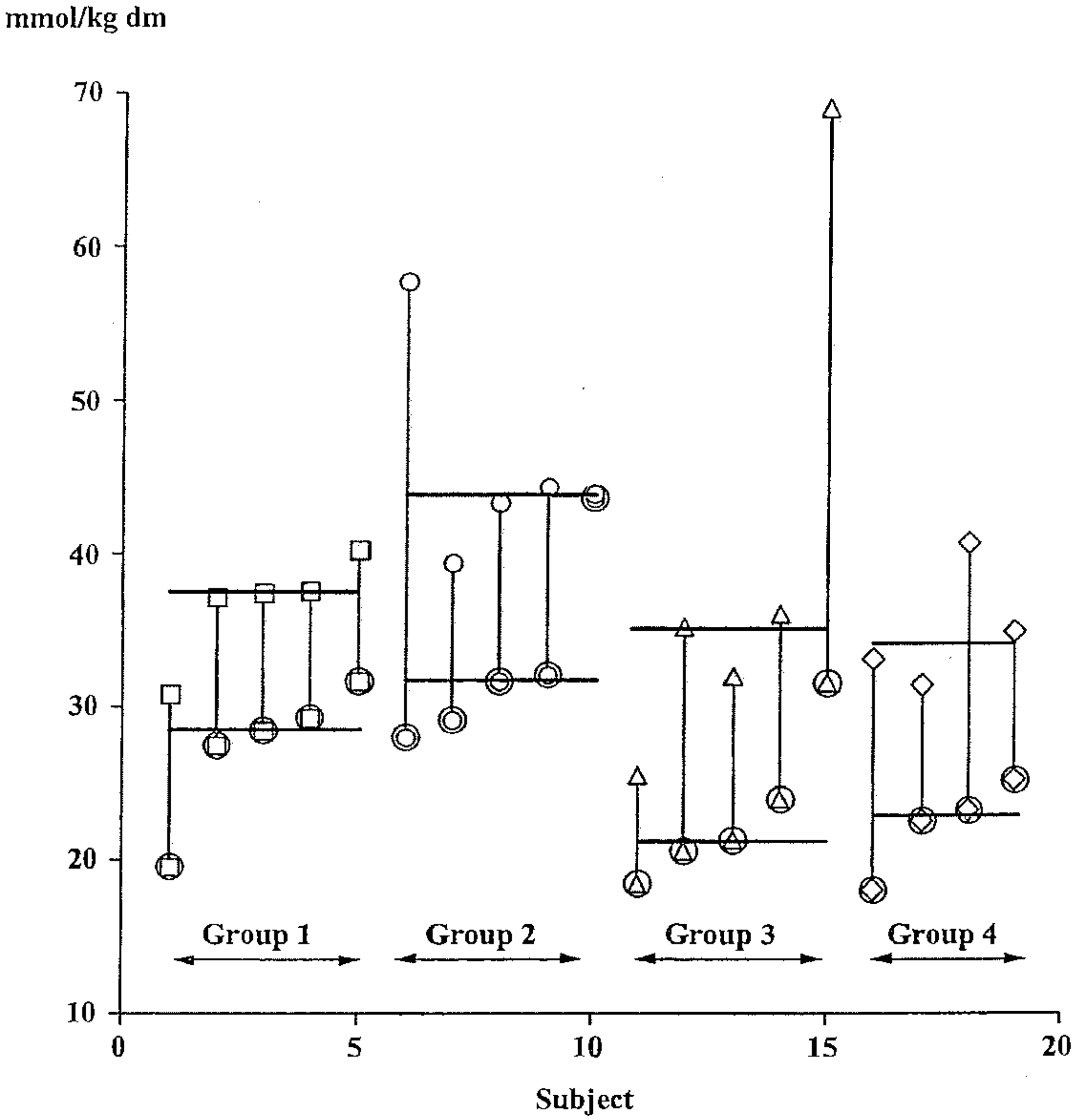

mmol/kg dm
70
60
50
40
30
20
10
0
5
10
15
20
Group 1
Group 2
Group 3
Group 4
Subject

FIG. 12

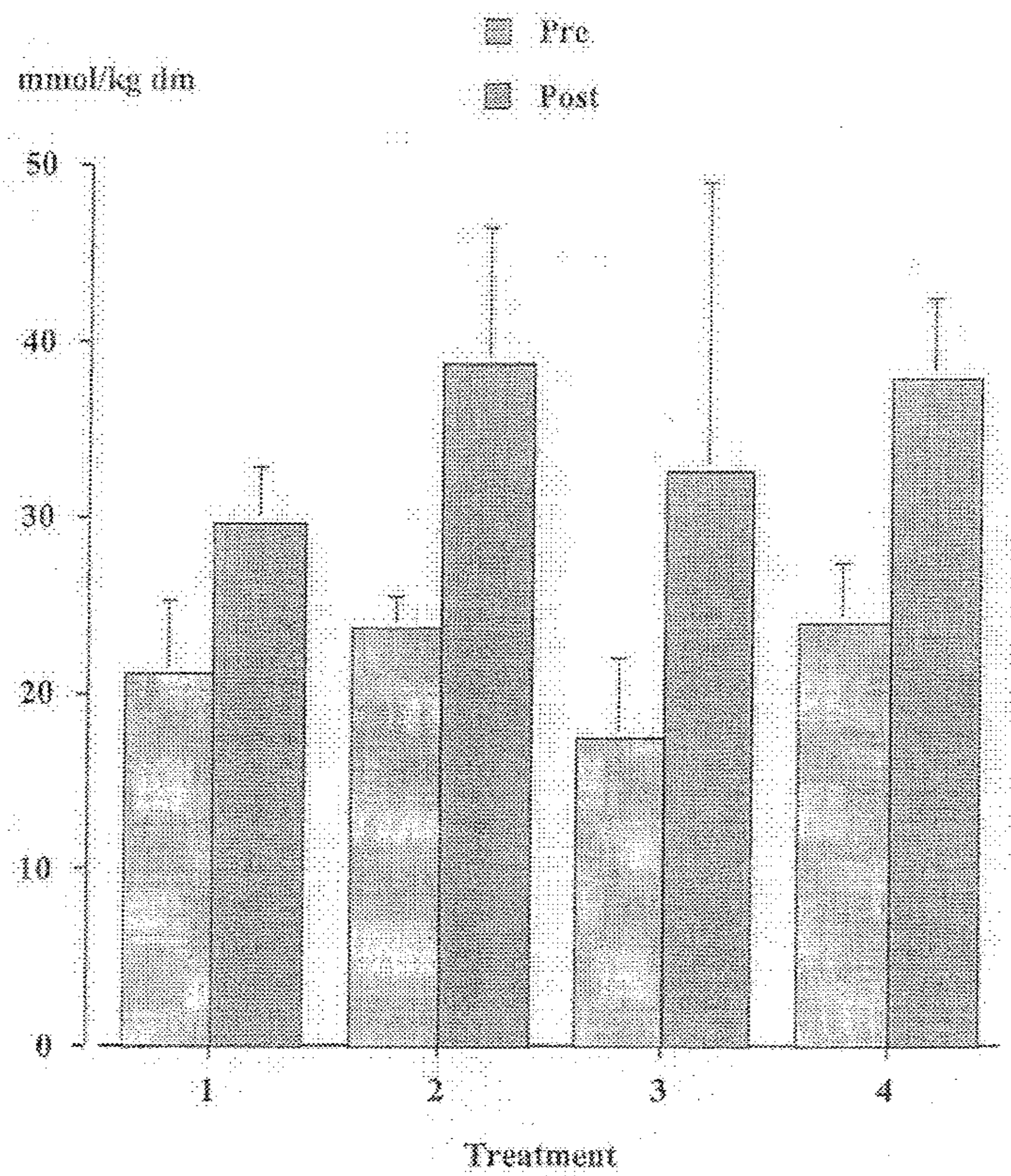


FIG. 13

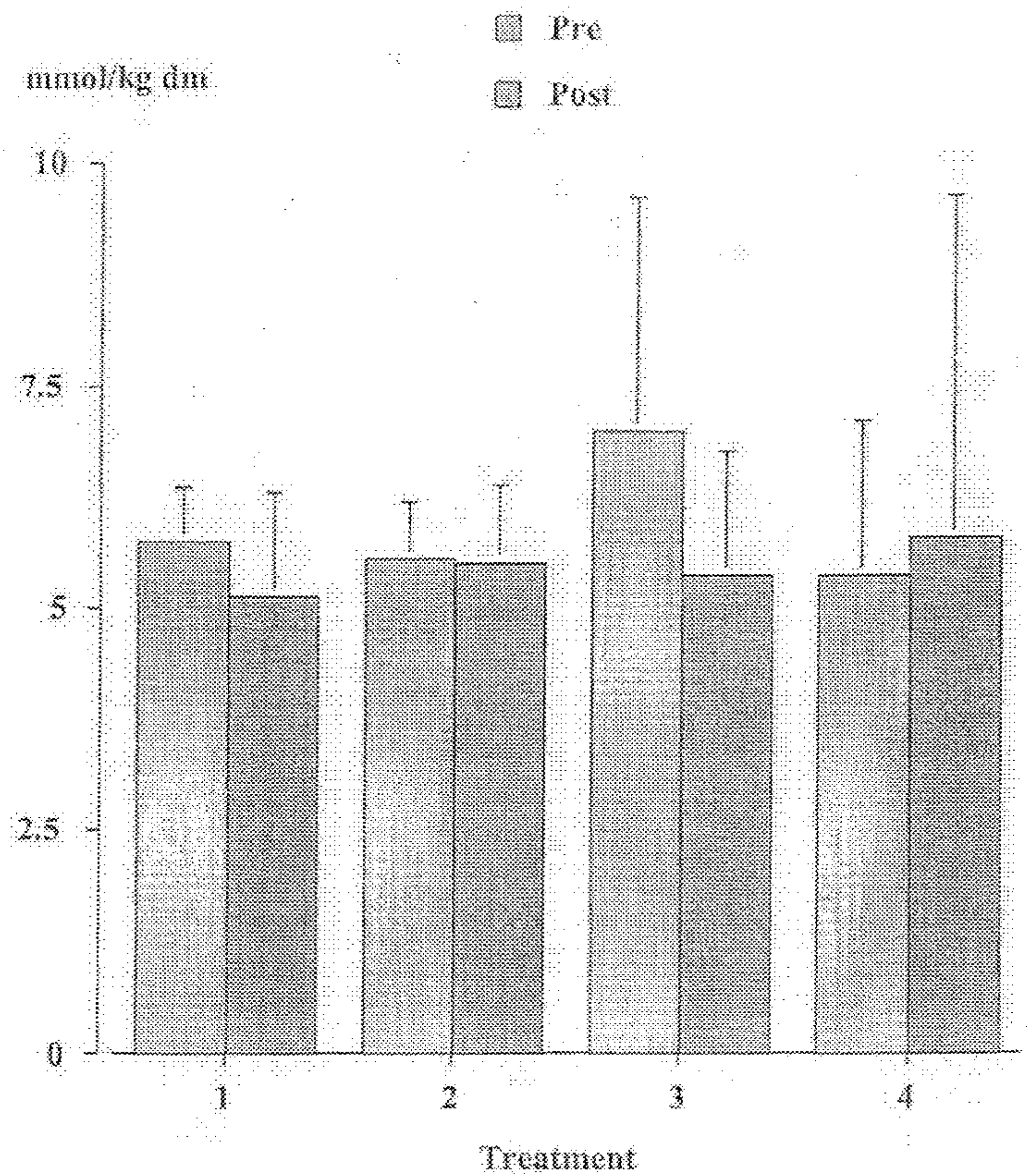


FIG. 14

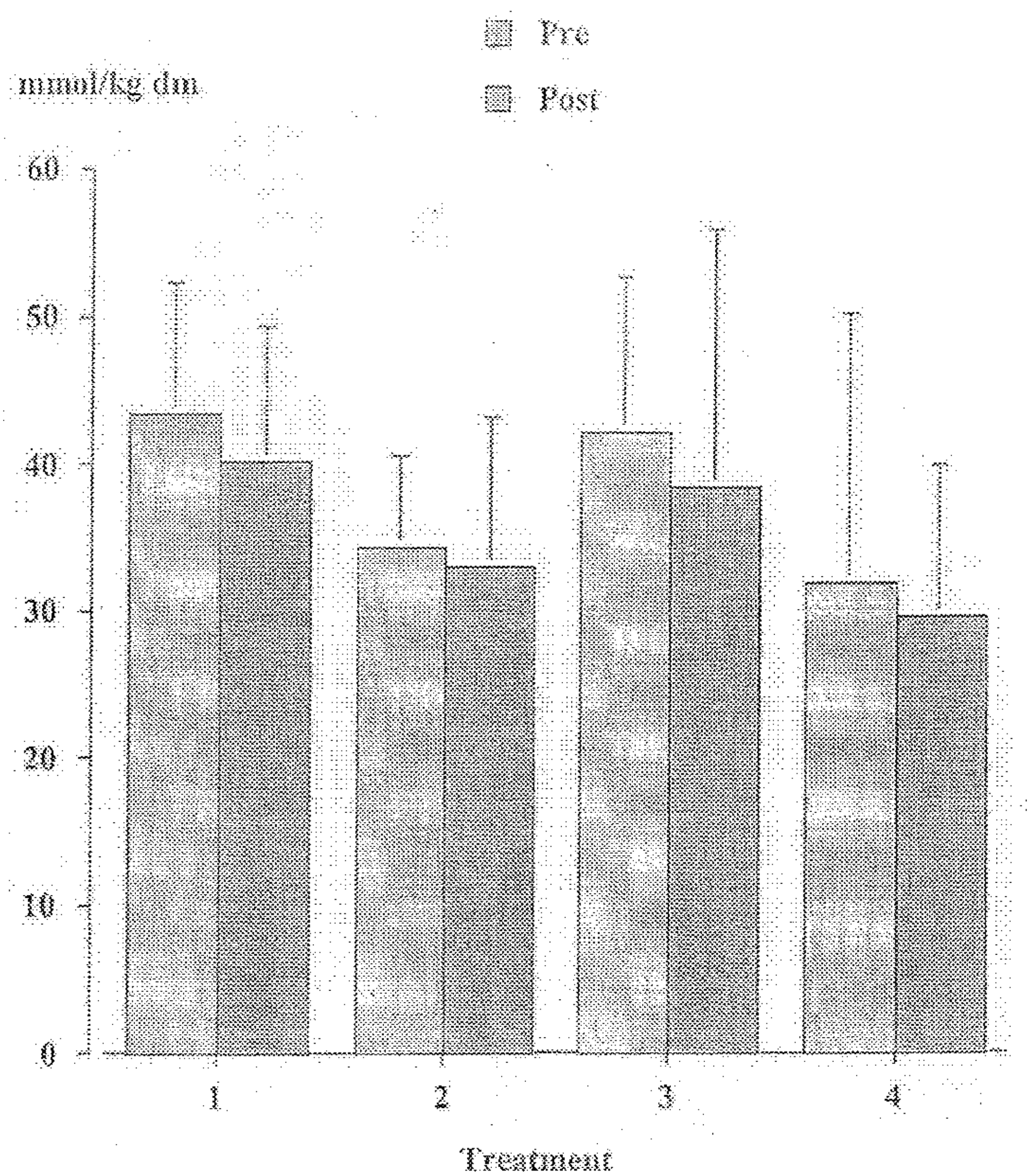


FIG. 15

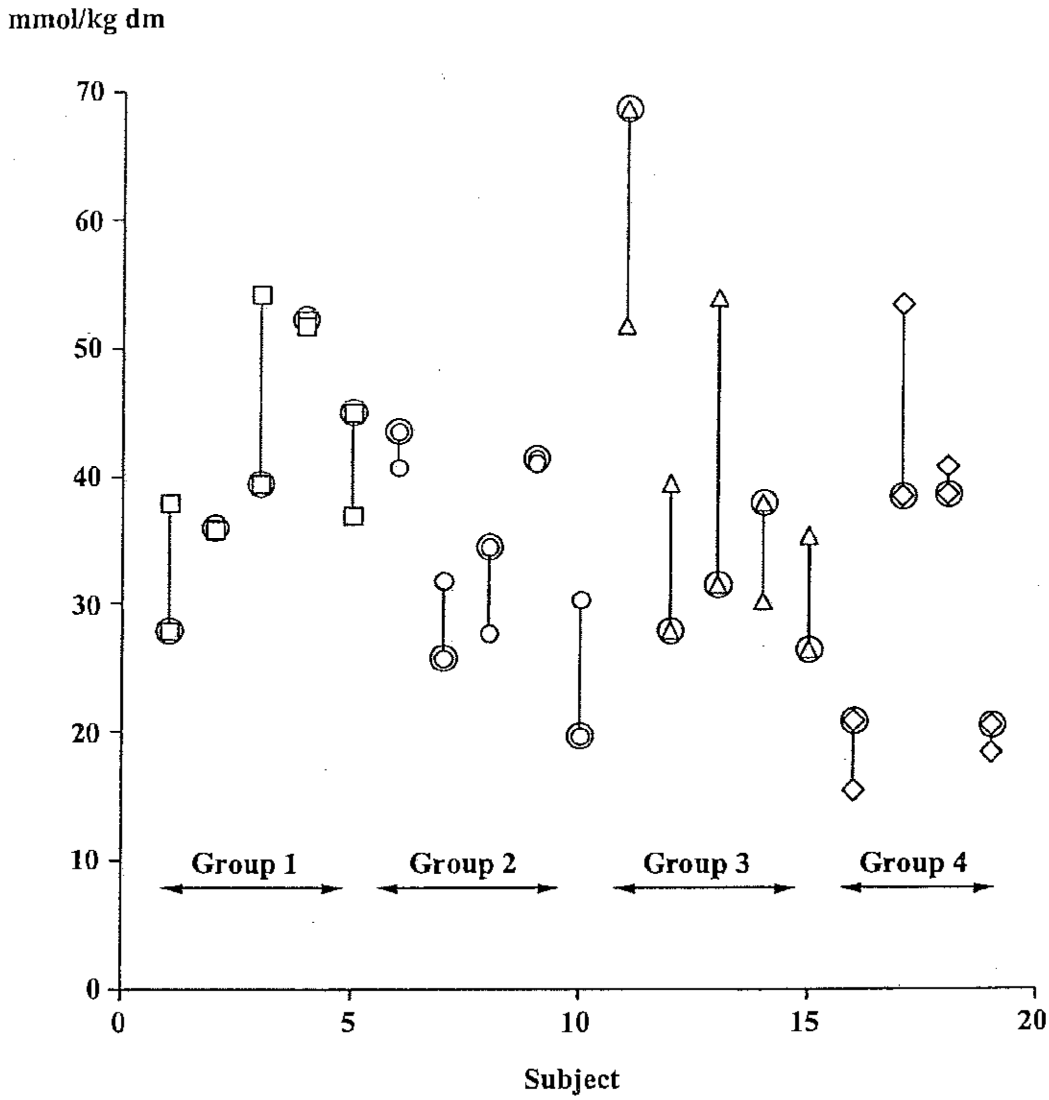

mmol/kg dm
70
60
50
40
30
20
10
0
Group 1
Group 2
Group 3
Group 4
0
5
10
15
20
Subject

FIG. 16

Table 9

TREATMENT		DOSING TIMES								AVG DOSE (mg x times)	PER DAY	
		9am	10am	11am	12noon	3pm	4pm	5pm	6pm		GIVEN	as ß-Ala
1 Beta alanine (ß-Ala) n = 5	Week 1		800mg		800mg		800mg		800mg	800 x 4	3.2g	3.2g
	2		800mg		800mg		800mg		800mg	800 x 4	3.2g	3.2g
	3		800mg		800mg		800mg		800mg	800 x 4	3.2g	3.2g
	4		800mg		800mg		800mg		800mg	800 x 4	3.2g	3.2g
										Total 90g ß-Ala in 4W		
2 Beta alanine (ß-Ala) n = 5	Week 1	800mg	400mg	400mg	400mg	800mg	400mg	400mg	400mg	500 x 8	4.0g	4.0g
	2	800mg	400mg	400mg	800mg	800mg	400mg	400mg	800mg	600 x 8	4.8g	4.8g
	3	800mg	400mg	800mg	800mg	800mg	400mg	800mg	800mg	700 x 8	5.6g	5.6g
	4	800mg	800mg	800mg	800mg	800mg	800mg	800mg	800mg	800 x 8	6.4g	6.4g
										Total 146g ß-Ala in 4W		
3 Carnosine (C) n = 5	Week 1	1500mg	1500mg	1000mg	1000mg	1500mg	1500mg	1000mg	1000mg	1250 x 8	10g	4.0g
	2	1500mg	1500mg	1500mg	1500mg	1500mg	1500mg	1500mg	1500mg	1500 x 8	12g	4.8g
	3	2000mg	1500mg	1500mg	2000mg	2000mg	1500mg	1500mg	2000mg	1750 x 8	14g	5.6g
	4	2000mg	2000mg	2000mg	2000mg	2000mg	2000mg	2000mg	2000mg	2000 x 8	16g	6.4g
										Total 364g C in 4W (145g ß-Ala)		

FIG. 17

METHODS AND COMPOSITIONS FOR INCREASING THE ANAEROBIC WORKING CAPACITY IN TISSUES

RELATED APPLICATIONS

This application is a continuation of U.S. patent application Ser. No. 10/717,217, filed Nov. 18, 2003, now U.S. Pat. No. 7,504,376, which claims the benefit of priority under 35 U.S.C. Section 119(e) of U.S. Provisional Application No. 60/462,238 filed Apr. 10, 2003 and that is a continuation-in-part (CIP) of U.S. application Ser. No. 10/209,169, filed Jul. 30, 2002, now U.S. Pat. No. 6,680,294, which is a continuation of U.S. application Ser. No. 09/757,782, filed Jan. 9, 2001, now U.S. Pat. No. 6,426,361, which is a continuation of U.S. application Ser. No. 09/318,530, filed May 25, 1999, now U.S. Pat. No. 6,172,098, which is a divisional of U.S. application Ser. No. 08/909,513, filed Aug. 12, 1997, now U.S. Pat. No. 5,965,596, which claims the benefit of foreign priority under 35 USC 119 to United Kingdom application nos. 9621914.2, filed Oct. 21, 1996, now terminated and 9616910.7, filed Aug. 12, 1996, now terminated. The aforementioned applications are incorporated by reference in their entirety.

TECHNICAL FIELD

This invention relates to the fields of pharmaceuticals and physiology. In one aspect, the invention provides methods for increasing the buffering capacity of mnscles and decreasing muscle fatigue. The invention also provides methods and compositions for increasing the anaerobic working capacity of muscle and other tissues.

BACKGROUND

Natural food supplements are typically designed to compensate for reduced levels of nutrients in the modern human and animal diet. In particular, useful supplements increase the function of tissues when consumed. It can be particularly important to supplement the diets of particular classes of animals whose normal diet may be deficient in nutrients available only from meat and animal products (e.g., human vegetarians and other animals who consume an herbivorous diet).

For example, in the sporting and athletic community, natural food supplements which specifically improve athletic ability are increasingly important, such as snpplements that promote or enhance physical prowess for leisure or employment purposes. In another example, anaerobic (e.g., lactate-producing) stress can cause the onset of fatigue and discomfort that can be experienced with intense exercise (e.g., continuous or intermittent sprinting in soccer or ice-hockey), where oxygen availability may be limited (e.g., peripheral vascular disease, free diving or synchronized swimming) and with aging. Anaerobic stress can also result from prolonged submaximal isometric exercise when the local circulation is partially or totally occluded by the increase in intra-muscular pressure (e.g., during rock climbing). Excessive lactate production can result in the acidification of the intracellular environment.

Creatine (i.e., N-(aminoiminomethyl)-N-glycine, N-amidinosarcosine, N-methyl-N-guanylglycine, or methylglycocyamine) is found in large amounts in skeletal muscle and other "excitable" tissues (e.g., smooth muscle, cardiac muscle, or spermatozoa) characterized by a capacity for high and variable energy demand. Creatine is converted into phosphorylcreatine in energy-generating biochemical pathways within cells. In mammalian skeletal muscle, the typical combined content of creatine (i.e., creatine and phosphorylcreatine) may vary from less than 25 to about 50 mmol per kilogram fresh muscle (i.e., 3.2 to 6.5 grams per kilogram fresh muscle).

Creatine is formed in the liver and taken up into tissues, such as muscle, by means of an active transport system. Creatine synthesis in the body may also be augmented by the ingestion of creatine present in meat (e.g., 5-10 milligrams per kilogram body weight per day in the average meat-eating human and approximately zero in a vegetarian diet).

During sustained intense exercise, or exercise sustained under conditions of local hypoxia, the accumulation of hydronium ions formed during glycolysis and the accumulation of lactate (anaerobic metabolism) can severely reduce the intracellular pH. The reduced pH can compromise the function of the creatine-phosphorylcreatine system. The decline in intracellular pH can affect other functions within the cells, such as the function of the contractile proteins in muscle fibers.

Dipeptides (also referred to herein as peptides) of beta-alanine and histidine, and their methylated analogues, which include carnosine (beta-alanyl-L-histidine), anserine (beta-alanyl-L-1-methylhistidine), or balenine (beta-alanyl-L-3-methylhistidine), are present in the muscles of humans and other vertebrates. Carnosine is found in appreciable amounts in muscles of, for example, humans and equines. Anserine and camosine are found in muscles of, for example, canines, camelids and numerous avian species. Anserine is the predominant beta-alanylhistidine dipeptide in many fish. Balenine is the predominant beta-alanylhistidine dipeptide in some species of aquatic mammals and reptiles. In humans, equines, and camelids, the highest concentrations of the beta-alanylhistidine dipeptides are found in fast-contracting glycolytic muscle fibers (type IIA and IIB) which are used extensively during intense exercise. Lower concentrations are found in oxidative slow-contracting muscle fibers (type I). See, e.g., Dunnett, M. & Harris, R. C. Equine Vet. J., Suppl. 18, 214-217 (1995). It is known that camosine contributes to hydronium ion buffering capacity in different muscle fiber types, and up to 50% of the total in equine type II fibers.

SUMMARY

The invention provides methods of increasing anaerobic working capacity in a tissue, comprising the following steps: (a) providing a beta-alanylhistidine dipeptide and a glycine, an insulin, an insulin mimic, or an insulin-action modifier; and (b) administering the beta-alanine and at least one of the glycine, insulin mimic, or insulin-action modifier to the tissue in an amount effective to increase beta-alanylhistidine dipeptide synthesis in the tissue, thereby increasing the anaerobic working capacity in the tissue. The invention provides methods of regulating hydronium ion concentrations in a tissue comprising the following steps: (a) providing a beta-alanylhistidine dipeptide and a glycine, an insulin an insulin mimic, or an insulin-action modifier; and (b) administering the beta-alanine and at least one of the glycine, insulin mimic, or insulin-action modifier to the tissue in an amonnt effective to increase the hydronium ion concentration in the tissue.

In one aspect of the methods, the step of administering the beta-alanine and at least one of the glycine, insulin mimic, or insulin-action modifier to the tissue comprises oral administration, administration to a blood or blood plasma or a combination thereof. The beta-alanylhistidine dipeptide can comprise a camosine, an anserine, or a balenine, or analogs or mimetics thereof.

The invention provides compositions comprising a mixture of a glycine, an insulin, an insulin mimic or an insulin-action modifier, and a composition comprising an amino acid or an active derivative thereof selected from the group consisting of a beta-alanine, a chemical derivative of beta-alanine and a peptide comprising a beta-alanine or analogs thereof. In one aspect, the beta-alanine comprises a beta-alanylhistidine dipeptide, such as a carnosine, an anserine or a balenine or analogs thereof. The compositions can further comprise at least a creatine or a carbohydrate.

In one aspect, the insulin mimic comprises a D-pinitol (3-O-methyl-chiroinositol), a 4-hydroxy isoleucine, a demethyl-asterriquinone B-1 compound, an alpha lipoic acid, an R-alpha lipoic acid, a guanidiniopropionic acid, a vanadium compound, a vanadium complex or a synthetic phosphoinositolglycan peptide. The insulin-action modifier can be a sulphonylurea, a thiazolidinedione or a biguanide.

In alternative aspects, the composition is a pharmaceutical composition, a dietary supplement or a sports drink. The dietary supplement or sports drink can be a supplement for humans. The pharmaceutical composition can be formulated for humans.

The invention provides compositions comprising at least 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 or more grams of a peptide or an ester comprising a beta-alanine or analogs or mimetics thereof. The invention provides compositions comprising at least about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.1, 6.2, 6.3, 6.4, 6.5 or more grams of a peptide or an ester comprising a beta-alanine (or analogs or mimetics thereof) in an injectable form. In one aspect, the peptide comprises a beta-alanylhistidine dipeptide, such as a carnosine, an anserine or a balenine, or analogs or mimetics thereof.

The invention provides compositions formulated for humans comprising at least 200, 225, 250, 275, 300, 325, 350, 400, 425, 450, 475, 500, 525, 550, 575, 600, 625, 650, 675, 700, 750, 775, 800, 825, 850, 875, 900, 925, 950, 975 or 1000 or more mg of a beta-alanine or beta-alanine analogs or mimetics. In one aspect, the composition is formulated in an ingestible or an injectable formulation. The ingestible formulation can be a drink, a gel, a food or a tablet. The peptide can comprise a beta-alanylhistidine dipeptide, such as a carnosine, an anserine or a balenine, or analogs or mimetics thereof.

The invention provides methods of increasing the anaerobic working capacity of a tissue in a subject comprising the following steps: (a) providing a composition comprising (i) a mixture of a glycine, an insulin, an insulin mimic or an insulin-action modifier, and a composition comprising an amino acid or an active derivative thereof selected from the group consisting of a beta-alanine, a chemical derivative of beta-alanine and a peptide comprising a beta-alanine, or analogs or mimetics thereof; (ii) at least 0.5 gram of a peptide or an ester comprising a beta-alanine in an injectable form; or, (iii) at least 200 mg of a beta-alanine; and (b) administering the composition to the subject in an amount effective to increase the anaerobic working capacity of the tissue. In one aspect, the total dosage of the beta-alanine for a 24-hour period is at least about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.1, 6.2, 6.3, 6.4, 6.5 or more grams. The total dosage of the beta-alanine for a 24-hour period can be between about 0.2 gram and about 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7.0 or more grams. The composition can be given over a period of at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 or more days. The composition can be given over a period of at least about 3 days to about two, three, four or more weeks. The beta-alanine can comprise a beta-alanylhistidine dipeptide, such as a carnosine, an anserine or a balenine, or analogs or mimetics thereof. The total dosage of the beta-alanylhistidine dipeptide over a 24 hour period can be at least about 0.5 gram, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.1, 6.2, 6.3, 6.4, 6.5 or more grams. The total dosage of the beta-alanylhistidine dipeptide over a 24 hour period can be greater than about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 or more grams. The total dosage of the beta-alanylhistidine dipeptide over a 24 hour period can be more than about 5 gram to about 16 gram. The composition can be administered in multiple doses. The composition can be administered at least two times to eight times in a 24-hour period. In one aspect, about 200 mg, 225, 250, 275, 300, 325, 350, 400, 425, 450, 475, 500, 525, 550, 575, 600, 625, 650, 675, 700, 750, 775, 800, 825, 850, 875, 900, 925, 950, 975 or 1000 mg of a beta-alanine (or analogs or mimetics thereof) and/or about 500 mg (or, about 200 mg, 225, 250, 275, 300, 325, 350, 400, 425, 450, 475, 500, 525, 550, 575, 600, 625, 650, 675, 700, 750, 775, 800, 825, 850, 875, 900, 925, 950, 975 or 1000 mg) of carnosine (or analogs or mimetics thereof) is administered about two to eight, or more, times a day (e.g., 2, 3, 4, 5, 6, 7, 8 or more times a day) over a period of several weeks. In one aspect, at least about 2 g of a beta-alanine or at least about 5 g of carnosine is administered about two to eight times a day over a period of about two, three or four days.

In one aspect, the amount of a composition of the invention administered is increased daily. The amount of the composition of the invention administered can be increased weekly. The composition can be administered in treatment periods that last for at least about four weeks.

While the invention is not limited by any particular mechanism of action, the invention provides methods of regulating hydronium ion concentration in tissue in a subject comprising the following steps: (a) providing a composition comprising (i) a mixture of a glycine, an insulin, an insulin mimic or an insulin-action modifier, and a composition comprising an amino acid or an active derivative thereof selected from the group consisting of a beta-alanine, a chemical derivative of beta-alanine and a peptide comprising a beta-alanine or analogs or mimetics thereof; (ii) at least 0.5 gram of a peptide or an ester comprising a beta-alanine in an injectable form; or, (iii) at least 200 mg of a beta-alanine; and (b) administering the composition to the subject in an amount effective to regulate the hydronium ion concentration in the tissue.

In one aspect, the invention features methods and compositions for increasing the anaerobic working capacity of muscle and other tissues. The methods and compositions of the invention provide for the simultaneous accumulation of creatine and/or beta-alanylhistidine dipeptides, or beta-alanine and L-histidine analogues, within a tissue in the body. The methods include ingesting or infusing compositions into the body. In one aspect, the compositions are mixtures of compounds capable of increasing the availability and uptake of creatine and of precursors for the synthesis and accumulation of beta-alanylhistidine dipeptides in human and animal tissue. The compositions of the invention can induce the synthesis and accumulation of beta-alanylhistidine dipeptides in a human or animal body when introduced into the body.

The compositions can include beta-alanine, chemical derivatives and analogs of beta-alanine such as esters of beta-alanine, peptides of beta-alanine, such as carnosine, anserine, and balenine, as well as analogues thereof. The compositions may also include L-histidine and mixtures thereof. Each of

the beta-alanine and/or L-histidine can be formulated or administered as individual amino acids, or, as components of dipeptides (e.g., carnosine, anserine, and/or balenine), oligopeptides, or polypeptides. The beta-alanine, L-histidine, carnosine, anserine, and/or balenine, or peptides of beta-alanine can be active derivatives. An active derivative is a compound derived from, or is a precursor of, a substance and performs in the same or similar way in the body as the substance, or which is processed into the substance when placed into the body. Examples include, for example, esters and amides. Compositions can also include any one or more of a creatine, a carbohydrate, insulin, an insulin mimic, an insulin-action modifier or a glycine. The compositions of the invention can be used for the preparation of a dietary supplement (including, e.g., drinks, gels, foods) or pharmaceutical composition for humans or animals. The compositions of the invention can be used in any of the methods of the invention.

In one aspect, the invention features compositions for and a method of regulating hydronium ion concentrations in a tissue. The method includes the steps of providing an amount of beta-alanine to blood or blood plasma effective to increase beta-alanylhistidine dipeptide synthesis in a tissue and exposing the tissue to the blood or blood plasma, whereby the concentration of beta-alanylhistidine is increased in the tissue. The beta-alanylhistidine may be a carnosine, anserine, or a balenine. The method can include the step of providing an amount of L-histidine to the blood or blood plasma effective to increase beta-alanylhistidine dipeptide synthesis.

In another aspect, the invention features a method of increasing the anaerobic working capacity of a tissue. The method includes the steps of providing an amount of beta-alanine to blood or blood plasma effective to increase beta-alanylhistidine dipeptide synthesis in a tissue, providing an amount of L-histidine to the blood or blood plasma effective to increase beta-alanylhistidine dipeptide synthesis in a tissue, and exposing the tissue to the blood or blood plasma. The concentration of beta-alanylhistidine is increased in the tissue.

In alternative aspects, the methods can include the step of increasing a concentration of creatine in the tissue. The increasing step can include providing an amount of creatine to the blood or blood plasma effective to increase the concentration of creatine in the tissue (e.g., by providing creatine to the blood or blood plasma).

The providing steps of the methods can include ingestion, infusion (e.g., injection) or a combination of ingestion and infusion, of a composition including an amount of beta-alanine, a peptide of beta-alanine such as carnosine, anserine and balenine which are hydrolyzed to their constituent amino acids on ingestion and are a source of beta-alanine for the body. Methods of the invention also include providing L-histidine, creatine, carbohydrate, insulin, insulin mimics, insulin-action modifiers and/or glycine.

In yet another aspect, the methods can include increasing a concentration of insulin in the blood or blood plasma. The concentration of insulin can be increased, for example, by injection of insulin. Methods of the invention can also include injection ingestion, or other modes of delivery, known to those of skill in the art, to a body (also referred to as a subject) of insulin mimics. Examples of insulin mimics include, but are not limited to, D-pinitol (3-O-methyl-chiroinositol), 4-hydroxy isoleucine, L783,281 (a demethyl-asterriquinone B-1 compound), alpha lipoic acid, R-alpha lipoic acid, guanidiniopropionic acid, vanadium compounds such as vanadyl sulfate or vanadium complexes such as peroxovanadium, and synthetic phosphoinositolglycans (PIG peptides). Additionally or alternatively, methods of the invention can include the use of insulin-action modifiers to enhance or inhibit the action of insulin in the body. Examples of insulin-action modifiers can include, but are not limited to, sulphonylureas, thiazolidinediones, and biguanides.

In still another aspect, the methods include providing glycine to a body. It is thought that glycine may suppress blood glucose release in the blood after ingestion of a meal. It may be that glycine enhances insulin sensitivity by promoting greater glucose uptake. Accordingly, the methods include providing glycine alone or in conjunction with insulin, insulin mimics or insulin-action modifiers in the compositions and methods of the invention. Glycine may be provided in various forms, for example, alone or in combination with other substances, such as in dietary supplements. Alternatively, glycine can be derived from other sources, such as gelatin.

The tissue referred to in the invention can be a skeletal muscle.

In one aspect, the invention provides compositions for practicing the methods of the invention. Accordingly, one aspect of the invention contemplates a composition having one or more active ingredient, including beta-alanine, beta-alanylhistidine peptides (or analogues or derivatives thereof), creatine, insulin, insulin mimics or insulin-action modifiers, glycine, and carbohydrate, to carry out the methods of the invention. The invention further contemplates the use of multiple compositions formulated to provide one or more active ingredient to the body for carrying out the methods of the invention.

Therefore, in an exemplary aspect, the invention features a composition consisting essentially of beta-alanine or a peptide source of beta-alanine, between about 39 and about 99 percent by weight of a carbohydrate, and up to about 60 percent by weight of water. The composition can include between about 0.1 and about 20 percent by weight of the beta-alanine (in the free or a bound form). The composition can include between about 0.1 and about 20 percent by weight of L-histidine.

The carbohydrate can be a simple carbohydrate (e.g., glucose).

In another aspect, the invention features a composition consisting essentially of beta-alanine or a peptide source of beta-alanine, between about 1 and about 98 percent by weight of a creatine source, and up to about 97 percent by weight of water. The composition includes between about 0.1 and about 98 percent by weight of the beta-alanine. The peptide source can include L-histidine and the composition can include between about 0.1 and about 98 percent by weight of L-histidine from this source.

The peptide source can be a mixture of amino acids, dipeptides, oligopeptides, polypeptides, or active derivatives thereof.

The composition can be a dietary supplement. The creatine source can be creatine monohydrate.

The concentrations of components in blood or blood plasma, including beta-alanine, can be increased by infusion (i.e., injection) or ingestion of an agent operable to cause an increase in the blood plasma concentration. The composition can be ingested in doses of between about 100 milligrams and about 800 grams or more per day. The doses can be administered in one part or multiple parts each day.

An increase of creatine and beta-alanylhistidine dipeptides in the muscles can increase the tolerance of the cells to an increase in hydronium ion production with anaerobic work and lead to an increase in endurance during exercise before the onset of fatigue. The compositions and methods can contribute to correcting the loss of beta-alanine, L-histidine, or creatine due to degradation or leaching of these constituents

during the cooking or processing of food. The compositions and methods can also contribute to correcting the absence of these components from a vegetarian diet.

The methods and compositions can be used to increase beta-alanylhistidine dipeptides in sportsmen, athletes, body-builders, synchronized swimmers, soldiers, elderly people, horses in competition, working and racing dogs, and game birds, to avoid or delay the onset of muscular fatigue.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other advantages and features of the invention will be apparent to the skilled artisan from the detailed description, drawings, and claims.

All publications, patents, patent applications cited herein are hereby expressly incorporated by reference for all purposes.

DESCRIPTION OF DRAWINGS

FIG. **1** is a graph depicting changes in the concentrations of beta-alanine in blood plasma of five horses, before and at 2 hour intervals following the feeding of beta-alanine and L-histidine (100 milligrams per kilogram body weight and 12.5 milligrams per kilogram body weight, respectively, three times per day) over a period of 30 days.

FIG. **2** is a graph depicting changes in the concentrations of L-histidine in blood plasma of five horses, before and at 2 hour intervals following the feeding of beta-alanine and L-histidine (100 milligrams per kilogram hody weight and 12.5 milligrams per kilogram body weight, respectively, three times per day) over a period of 30 days.

FIGS. **3***a*, **3***b*, **3***c*, **3***d*, **3***e* and **3***f* are graphs depicting the contrast in the changes in the concentrations of beta-alanine in blood plasma of six horses, before and at hourly intervals following the feeding of beta-alanine and L-histidine, as described in detail, below.

FIGS. **4***a*, **4***b*, **4***c*, **4***d*, **4***e* and **4***f* are graphs depicting the contrast in the changes in the concentrations of L-histidine in blood plasma of six horses, before and at hourly intervals following the feeding of beta-alanine and L-histidine, as described in detail, below.

FIG. **5** is a graph depicting the contrast in the changes in the mean concentrations of beta-alanine in equine blood plasma (n=6), before and at hourly intervals following the feeding of beta-alanine and L-histidine, as described in detail, below.

FIG. **6** is a graph depicting the contrast in the changes in the mean concentrations of L-histidine in equine blood plasma (n=6), before and at hourly intervals following the feeding of beta-alanine and L-histidine (100 milligrams per kilogram body weight and 12.5 milligrams per kilogram body weight, respectively, three times per day) on the first and last day of a 30 day period of dietary supplementation.

FIG. **7** is a graph depicting the correlation between the increase in 6 thoroughbred horses in the carnosine concentration in type II skeletal muscle fibers (the average of the sum of type IIA and IIB fibers) and the increase, between the 1st and 30th day of supplementation, in the area under the blood plasma beta-alanine concentration-time curve over the first 12 hours of the day ($AUC_{(0\text{-}12\ hr)}$).

FIG. **8** is graph depicting the mean results of the administration of beta-alanine, broth, or carnosine to test suhjects.

FIG. **9** is a graph depicting mean changes in plasma beta-alanine over nine hours of treatment.

FIG. **10** is a graph depicting the mean changes in plasma beta-alanine over 9 hours following the oral ingestion of 10 milligrams per kilogram body weight of beta-alanine.

FIG. **11** is a graph depicting the mean (n=6) plasma beta-alanine concentration over the 24 hours of Day 1 and Day 30 of the treatment period.

FIG. **12** is a graph depicting changes in muscle carnosine concentration pre and post treatment in different subjects. The red circles indicate the muscle concentrations prior to supplementation.

FIG. **13** is a graph depicting muscle concentration (mean±SD) of carnosine before and post supplementation in three different treatment groups.

FIG. **14** is a graph depicting muscle concentration (mean±SD) of histidine before and post supplementation in three different treatment groups.

FIG. **15** is a graph illustrating data showing muscle concentration (mean±SD) of taurine before and post supplementation in four different treatment groups.

FIG. **16** is a graph illustrating data showing muscle concentration (mean±SD) of taurine before and post supplementation in different subjects.

FIG. **17** illustrates a table of data, described in detail as Table 9, below.

Like reference symbols in the various drawings indicate like elements.

DETAILED DESCRIPTION

The invention provides compositions comprising beta-alanine, peptides of beta-alanine, analogues and derivatives thereof, beta-alanylhistidine dipeptides (e.g., carnosine, anserine, and halenine) and methods using these compositions for increasing the anaerobic working capacity of a tissue comprising providing an amount of beta-alanine to blood or blood plasma effective to increase beta-alanylhistidine dipeptide synthesis in a tissue. Beta-alanylhistidine dipeptides can include peptides of beta-alanine, such as carnosine, anserine, and balenine. In one aspect, they can have pKa values between approximately 6.8 and 7.1. In one aspect, they can be involved in the regulation of intra-cellular pH homeostasis during muscle contraction and the development of fatigue. The content of other substances involved in hydronium ion buffering, such as amino acid residues in proteins, inorganic and organic phosphates and bicarbonate, can be constrained by their involvement in other cell functions. In one aspect, the beta-alanylhistidine dipeptides provide an effective way of accumulating pH-sensitive histidine residues into a cell. Variations in the muscle beta-alanylhistidine dipeptide concentrations affect the anaerobic work capacity of individual athletes.

The beta-alanylhistidine dipeptides are synthesized within the body from beta-alanine and L-histidine. These precursors can be generated within the body or are made available via the diet, including from the breakdown of an ingested beta-alanylhistidine dipeptide. Within the body, beta-alanine is transported to tissues such as muscle. In a typical fed state, the concentration of beta-alanine is low in comparison with the concentration of L-histidine in human and equine blood plasma. These concentrations should be viewed in relation to the affinity of the carnosine synthesizing enzyme, carnosine synthetase, for its substrates as determined by the Michaelis Menten constant (Km). The Km for histidine is about 16.8 μM. The Km for beta-alanine is between about 1000 and 2300 μM. The low affinity of carnosine synthetase for beta-alanine, and the low concentration of beta-alanine in muscle, demonstrate that the concentration of beta-alanine in muscle is limiting to the synthesis of the beta-alanylhistidine dipeptides.

Increasing the amount of beta-alanylhistidine dipeptides within a muscle favorably affects muscular performance and

the amount of work that can be performed by the muscle. Accordingly, it is desirable to increase the synthesis and accumulation of beta-alanylhistidine dipeptides in a tissue in a human or animal body.

The synthesis and accumulation of beta-alanylhistidine dipeptides in a human or animal body can be increased by increasing creatine within the body, increasing the blood or blood plasma concentrations of beta-alanine, increasing the blood or blood plasma concentrations of beta-alanine and creatine, or increasing the blood or blood plasma concentrations of beta-alanine, L-histidine, and creatine. The increase in dipeptides can be simultaneous with the increase in beta-alanine concentrations.

In one aspect, the compositions and methods of the invention can be used to increase blood plasma concentrations of beta-alanine, L-histidine and/or creatine by ingestion or infusion of beta-alanine, peptides of beta-alanine, L-histidine, creatine, carnosine, anserine, and/or balenine and/or active derivatives or analogs thereof alone or in various combinations. The compositions of the invention can be administered orally, enterally, or parenterally. For example, compositions of the invention can be orally ingested or infused through the skin through a topical cream or a patch.

The composition can include carbohydrates (e.g., simple carbohydrates), insulin, or agents that stimulate the production of insulin. Compositions can also include glycine, insulin, insulin mimics, and/or insulin-action modifies.

The compositions can be a dietary supplement that can be ingested, injected, or absorbed through the skin. Preferably, the compositions can be administered in one or more doses per day. The beta-alanine dosage can be between about 1 milligram and about 200 milligrams per kilogram body weight or the dose of a peptide of beta-alanine (e.g. carnosine) from 2.5 milligrams to 500 milligrams per kilogram body weight. In one aspect, the total amount of beta-alanine (or other composition of the invention) administered can be at least 200 mg, from 200 mg to 5 g, or from 5 g or more per day for a human. A single dose of active ingredient, e.g., beta-alanine, carnosine, anserine, or balenine, or mixtures thereof, may be formulated to be in the amount about 200, 400, 800 mg or more. The creatine (e.g., creatine monohydrate) dosage, or dosage of other compositions of the invention, can be between about 5 milligrams to 200 milligrams per kilogram body weight. The L-histidine dosage, or dosage of other compositions of the invention, can be between about 1 milligram to 100 milligrams per kilogram body weight. The simple carhohydrate (e.g., glucose) dosage, or dosage of compositions of the invention, can be between about 0.5 and 2.0 grams per kilogram body weight.

In an 80 kilogram person, suitable dosages per day can be between 0.08 grams to 16.0 grams of beta-alanine or 200 milligrams to 40 grams of a peptide of beta-alanine, 0.4 grams to 16.0 grams of creatine monohydrate, 0.08 grams to 8.0 grams of L-histidine, or 40 grams to 160 grams of glucose or other simple carbohydrate. The composition can be in a solid form or a liquid form or in a suspension which can be ingested or infused into the body. The composition can be ingested by humans in an amount of between 0.08 grams and 1000 grams or more per day, which may be taken in one or more parts throughout the day. In animals, the daily intake will be adjusted hy hody weight.

In one aspect, the total amount of a peptide of beta-alanine, for example, carnosine, anserine or balenine that can be administered per day may be at least 500 mg, between about 500 mg to about 5 g, between about 5 g to abont 16 g, or greater than 16 g. A single dose of a peptide of beta-alanine creatine, anserine or balenine, or mixtures thereof, may be formulated to be in the amount of 0.5, 1, 1.5, or 2 g (for each, or all, or the peptides in a formulation comprising a mixture).

For humans and animals, the compositions can be:

(a) 1% to 99% by weight of beta-alanine or 1% to 99% by weight of a peptide of beta-alanine;
 1% to 99% by weight of creatine monohydrate; and
 0% to 98% by weight of water;

(b) 1% to 98% by weight of beta-alanine or 1% to 98% by weight of a peptide of beta-alanine;
 1% to 98% by weight of L-histidine;
 1% to 98% by weight of creatine monohydrate; and
 0% to 97% by weight of water;

(c) 1% to 20% by weight of beta-alanine or 1% to 20% by weight of a peptide of beta-alanine;
 39% to 99% by weight of glucose or other simple carbohydrate; and
 0% to 60% by weight of water; or

(d) 1% to 20% by weight of beta-alanine or 1% to 20% by weight of a peptide of beta-alanine;
 1% to 20% by weight of L-histidine;
 39% to 99% by weight of glucose or other simple carbohydrate; and
 0% to 60% by weight of water.

In one aspect, compositions are applied to a body for at least three days, from 3 days to 2 weeks, from 2 weeks to 4 weeks, or longer. In certain regimens, the daily dosages are gradually increased or decreased. This can be done daily, every couple of days, or weekly.

EXAMPLES

The following are specific examples of the methods and compositions for increasing the anaerobic working capacity of muscle and other tissues.

Example 1

The effect of supplementation of a normal diet with multiple daily doses of beta-alanine and L-histidine on the carnosine concentration in type I, IIA, and IIB skeletal muscle fibers of thoroughbred horses was assessed. Six experimental thoroughbred horses of normal health (three fillies and three geldings), aged 4 to 9 years, underwent one month (30 days) of dietary conditioning (pre-supplementation period) prior to the commencement of the supplementation period. During the dietary conditioning period each horse was fed a diet comprising 1 kilogram of pelleted feed (Spillers racehorse cubes) and 1 kilogram of soaked sugar beet pulp as a source of complex and simple carbohydrates, three times per day (at 08:30, 12:30, and 16:30, respectively). Soaked hay (3 kilograms dry weight) was also provided twice daily (at 09:00 and 17:00). Water was provided ad libitum.

During the supplementation period, an identical feeding regime was implemented. However, each hard feed meal was supplemented with beta-alanine and L-histidine (free base). Beta-alanine and L-histidine were mixed directly into the normal feed. Individual doses of beta-alanine and L-histidine were calculated according to body weight. Beta-alanine was administered at 100 milligrams per kilogram body weight and L-histidine at 12.5 milligrams per kilogram body weight. Dietary supplementation was begun on day 1 of the protocol and discontinued at the end of day 30. Heparinized blood samples (5 milliliters) were collected on days 1, 6, 18, 24, and 30. On day 1 and day 30, blood samples were collected prior to the first feed and at hourly intervals for a total of 12 hours each day. On the three intervening sampling days, blood was collected prior to the first feed and 2 hours after each subse-

quent feed. On the day before the start of supplementation (day 0) a muscle biopsy was taken, following application of local anesthesia of the skin, from the right middle gluteal muscle (m. gluteus medius) of each horse using a Bergstrom-Stille percutaneous biopsy needle. Subsequent muscle biopsies were collected immediately after the end of the supplementation period (day 31) as close as possible to the original sampling site. Clinical monitoring of the horses was performed daily. This comprised a visual examination and measurement of body weight, twice-daily measurement of rectal temperature, and weekly blood sampling for clinical biochemistry and hematology. During the course of the study, the horses received no formal training or exercise, although they were allowed one hour of free exercise each day.

Fragments of individual muscle fibers dissected from freeze-dried muscle biopsies were characterized as either type I, IIA or IIB by histochemical staining for myosin ATPase activity at pH 9.6 following pre-incubation at pH 4.5 by a modification of the method described in, Kaiser and Brook, *Arch. Neurol.*, 23:369-379 (1970).

Heparinized blood plasma samples were extracted and analyzed for beta-alanine and L-histidine concentrations by high-performance liquid chromatography (HPLC). Individual weighed muscle fibers were extracted and analyzed for carnosine by HPLC according to the method described in, Dunnett and Harris, "High-performance liquid chromatographic determination of imidazole dipeptides, histidine, 1-methylhistidine and 3-methylhistidine in muscle and individual muscle fibers," *J. Chromatogr. B. Biomed. Appl.*, 688: 47-55 (1997).

Differences in carnosine concentrations within fiber types before and after supplementation were established within horses using one-way analysis of variance (ANOVA). In instances where differences were detected, significance was determined using a multiple comparison test (Fisher's PLSD).

No palatability problems were encountered with the addition of beta-alanine and L-histidine to the feed. No adverse physiological or behavioral effects of the supplemented diet were observed in any of the horses during the thirty days of supplementation. No significant changes in body weight were recorded, and rectal temperatures remained within the normal range. No acute or chronic changes in clinical biochemistry or hematology were observed. Beta-alanine was not detected in the plasma of any of the horses prior to the start of supplementation. The lower limit of quantitation for beta-alanine in plasma by the assay used was 3 micromolar (μM). Plasma L-histidine concentrations in the six horses prior to the start of supplementation were between 36.6 and 54.4 μM.

Individual changes in blood plasma beta-alanine and L-histidine concentrations for five of the six horses over all the sampling days are shown in FIGS. 1 and 2, respectively. There was a trend towards an increase in the pre-feeding concentrations of blood plasma beta-alanine and L-histidine with increasing time of supplementation. Furthermore, over the thirty day supplementation period, the blood plasma concentration response to supplementation was also increased. The response was greater for beta-alanine.

Comparisons of the changes in blood plasma beta-alanine and L-histidine concentrations prior to the first feed of the day, and hourly thereafter between the first and last days of the supplementation period, for the six individual horses, are shown in FIGS. 3*a* and 3*b*, and FIGS. 4*a* and 4*b*, respectively. FIGS. 3*a*, 3*b*, 3*c*, 3*d*, 3*e* and 3*f* are graphs depicting the contrast in the changes in the concentrations of beta-alanine in blood plasma of six horses, before and at hourly intervals following the feeding of beta-alanine and L-histidine (100 milligrams per kilogram body weight and 12.5 milligrams per kilogram body weight, respectively, three times per day) on the first and last day of a 30 day period of dietary supplementation. FIGS. 4*a*, 4*b*, 4*c*, 4*d*, 4*e* and 4*f* are graphs depicting the contrast in the changes in the concentrations of L-histidine in blood plasma of six horses, before and at hourly intervals following the feeding of beta-alanine and L-histidine (100 milligrams per kilogram body weight and 12.5 milligrams per kilogram body weight, respectively, three times per day) on the first and last day of a 30 day period of dietary supplementation. FIG. 5 is a graph depicting the contrast in the changes in the mean concentrations of beta-alanine in equine blood plasma (n=6), before and at hourly intervals following the feeding of beta-alanine and L-histidine (100 milligrams per kilogram body weight and 12.5 milligrams per kilogram body weight, respectively, three times per day) on the first and last day of a 30 day period of dietary supplementation. The mean (SD) changes (n=6) in blood plasma beta-alanine concentration over time during the 24 hours of the first (day 1) and last (day 30) days of the supplementation period are contrasted in FIG. 5. The area under the mean blood plasma beta-alanine concentration versus time curve over 24 hours ($AUC_{(0-24\ hr)}$) was much greater on day 30 of the supplementation.

The mean (SD) changes (n=6) in blood plasma L-histidine concentration over time during the 24 hours of the first (day 1) and last (day 30) days of the supplementation period are contrasted in FIG. 6. The area under the mean blood plasma beta-alanine concentration vs. time curve over 24 hours ($AUC_{(0-24\ hr)}$) was greater on day 30 of the supplementation. The greater AUC for blood plasma beta-alanine on the last day of supplementation (day 30) in contrast to the first day of supplementation (day 1) suggests the increased uptake of beta-alanine from the equine gastro-intestinal tract with progressive supplementation. A similar effect was observed for changes in blood plasma L-histidine concentration during the supplementation period. Peak blood plasma concentrations of beta-alanine and L-histidine occurred approximately one to two hours post-feeding in each case.

A total of 397 individual skeletal muscle fibers (192 pre-supplementation; 205 post-supplementation) from the six horses were dissected and analyzed for carnosine. Mean (SD) carnosine concentration, expressed as millimoles per kilogram dry weight (mmol kg^{-1} dw), in pre- and post-supplementation type I, IIA, and IIB skeletal muscle fibers from the six individual horses are given in Table 1 where n is the number of individual muscle fibers analyzed. Following thirty days of beta-alanine and L-histidine supplementation the mean carnosine concentration was increased in type IIA and IIB fibers in all six horses. These increases were statistically significant in seven instances. The increase in mean carnosine concentration in type IIB skeletal muscle fibers was statistically significant in five out of six horses. The increase in mean carnosine concentration in type IIA skeletal muscle fibers was statistically significant in two out of six horses.

TABLE 1

Horse	Day	Type I	n	Type IIA	n	Type IIB	n
6	0	32.3 (14.5)	3	72.1 (47.7)	11	111.8 (22.8)	14
	31			16.2 (20.9)	17	117.7 (38.7)	12
5	0	59.5 (3.9)	2	102.6 (12.7)	12	131.2 (26.6)	26
	31	55.5	1	112.2 (17.1)	18	153.3 (28.0)**	22
4	0	44.8 (6.6)	4	59.9 (19.5)	13	108.6 (41.5)	19
	31	37.0 (9.3)	2	88.0 (34.2)*	17	152.4 (65.0)*	19
1	0	56.7 (5.3)	2	88.5 (20.9)	15	101.3 (15.2)	13
	31	57.8	1	96.1 (17.3)	19	14.3 (13.3)*	11
2	0	—		89.6 (16.2)	13	104.2 (22.2)	14
	31	65.9 (13.2)	4	102.2 (22.1)	18	142.0 (35.4)***	12
3	0	30.9 (4.0)	2	85.1 (20.3)	6	113.5 (20.4)	23
	31	—		105.0 (17.6)*	23	135.4 (24.9)*	9
Mean	0	44.8	13	83.0	70	111.8	109
	31	54.1	8	96.6*	112	135.9**	85

*significantly different to pre-supplementation, $p < 0.05$
**significantly different to pre-supplementation, $p < 0.01$
***significantly different to pre-supplementation, $p < 0.005$

The absolute (e.g. mmol kg^{-1} dw) and percentage increases in the mean carnosine concentrations in type IIA and IIB skeletal muscle fibers from the six horses are listed in Table 2.

TABLE 2

Horse	Type IIA Absolute increase	Type IIA % increase	Type IIB Absolute increase	Type IIB % increase
6	4.1	5.7	5.6	5.3
5	9.6	9.4	22.1	16.8
4	28.1	46.9	43.8	40.3
1	7.6	8.6	13.0	12.8
2	12.6	14.1	37.8	36.3
3	19.9	23.4	21.9	19.3
Mean	13.6	18.0	24.1	21.8

It was observed that the individual horses which showed the greater increase in muscle carnosine concentration following thirty days of supplementation also demonstrated the greater increase in blood plasma beta-alanine AUC between day 1 and day 30 of the supplementation period. Referring to FIG. 7, a significant correlation ($r=0.986$, $p<0.005$) for five of the six horses was observed between the increase in mean carnosine concentration, averaged between type IIA and IIB skeletal muscle fibers and the increase, between the 1st and 30th day of supplementation, in blood plasma beta-alanine AUC, over the first 12 hours($AUC_{(0\text{-}12\ hr)}$). Only five horses were used to calculate the regression line. Horse 6 (filled circle) showed no appreciable increase in blood plasma beta-alanine concentration greater than that observed on day 1 until the last day of supplementation. This was unlike the other five horses, which showed a progressive increase with each sampling day. For this reason horse 6 was excluded from the calculation of the regression equation.

Increases in muscle carnosine concentration following thirty days of supplementation with beta-alanine and L-histidine will cause a direct increase in total muscle buffering capacity. This increase can be calculated by using the Henderson-Hasselbach Equation. Calculated values for the increases in muscle buffering capacity in type IIA and IIB skeletal muscle fibers in the six thoroughbred horses are shown in Table 3.

TABLE 3

Horse	Day	Type IIA βmcar	Type IIA βmtotal	Type IIA Δβmtotal (%)	Type IIB βmcar	Type IIB βmtotal	Type IIB Δβmtotal (%)
6	0	23.9	93.9	+1.5	37.1	107.1	+1.8
	31	25.3	95.3		39.0	109.0	
5	0	34.0	104.0	+3.1	43.5	113.5	+6.4
	31	37.2	107.2		50.8	120.8	
4	0	19.9	89.9	+10.3	36.0	106.0	+13.7
	31	29.2	99.2		50.5	120.5	
1	0	29.3	99.3	+2.6	33.6	103.6	+4.2
	31	31.9	101.9		37.9	107.9	
2	0	29.7	99.7	+4.2	34.5	104.5	+12.1
	31	33.9	103.9		47.1	117.1	
3	0	28.2	98.2	+6.7	37.6	107.6	+6.8
	31	34.8	104.8		44.9	114.9	
Mean	0	27.5	97.5	+4.7	37.1	107.1	+7.5
	31	32.1	102.1		45.0	115.0	

Example 2

The effect of supplementation of a normal diet with single and multiple daily doses of beta-alanine in free or peptide bound form on the beta-alanine and beta-alanyl dipeptide concentrations of plasma of humans was assessed. The plasma concentration of beta-alanine in six normal subjects following the consumption of a broth delivering approximately 40 milligrams per kilogram body weight of beta-alanine was monitored. Doses of 10 and 20 milligrams per kilogram body weight of beta-alanine were also given.

2.5 milliliter venous blood samples were drawn through an indwelling catheter at 10 minute intervals for the first 90 minutes and then after 120, 180, 240 and 360 minutes. The blood samples were dispensed into tubes containing lithium-heparin as an anti-coagulant. The catheter was maintained by flushing with saline. Plasma samples were analyzed by HPLC according to the method described in Jones & Gilligan (1983) *J. Chromatogr.* 266:471-482 (1983).

Table 4 summarizes the allocation of treatments during the beta-alanine absorption study. The estimated equivalent doses of beta-alanine are presented in Table 4.

TABLE 4

Subject	Age yrs	Weight kg	Broth 40 mg/kg bwt	β-ala 0 mg/kg bwt	β-ala 10 mg/kg bwt	β-ala 20 mg/kg bwt	β-ala 40 mg/kg bwt	Carnosine 20 mg/kg bwt
1	53	76	+			+	+	+
2	33	60	+			+	+	
3	29	105	+	+	+	+		
4	31	81	+	+	+		+	
5	30	94	+	+	+		+	
6	25	65	+	+	+	+		

The broth was prepared as follows. Fresh chicken breast (skinned and boned) was finely chopped and boiled for fifteen minutes with water (1 liter for every 1.5 kg of chicken). Residual chicken meat was removed by course filtration. The filtrate was flavored by the addition of carrot, onion, celery, salt, pepper, basil, parsley and tomato puree, and reboiled for a further fifteen minutes and then cooled before final filtration through fine muslin at 4° C. The yield from 1.5 kilograms of chicken and one liter of water was 870 mL of broth. A portion of the stock was assayed for the total beta-alanyl-dipeptide content (e.g., carnosine and anserine) and beta-alanine. Typical analyses were:

total beta-alanyl-dipeptides	74.5 mM
free beta-alanine	5.7 mM

The six male test subjects were of normal health and between 25-53 years of age, as shown in Table 4. The study commenced after an overnight fast (e.g., a minimum of 12 hours after the ingestion of the last meat containing meal). Subjects were given the option to consume a small quantity of warm water prior to the start of the study. Catheterization was begun at 08:30 and the study started at 09:00.

As a control, 8 milliliters per kilogram body weight of water was ingested (e.g., 600 mL in a subject weighing 75 kilograms).

In one session, 8 milliliters per kilogram body weight of broth containing approximately 40 milligrams per kilogram body weight of beta-alanine (e.g., in the form of anserine and carnosine) was ingested. For a subject weighing 75 kilograms, this amounted to the ingestion of 600 milliliters of broth containing 3 grams of beta-alanine. In another session, 3 milliliters per kilogram body weight of a liquid containing the test amount of beta-alanine with an additional 5 milliliters per kilogram body weight of water was ingested. In all sessions, subjects additionally consumed a further 8 milliliters per kilogram body weight of water (in 50 mL portions) during the period of 1 to 2 h after ingestion. A vegetarian pizza was provided after 6 hours. An ordinary diet was followed after 8 hours.

Plasma concentration curves following each treatment are depicted graphically in FIG. **8**. Mean results of the administration of beta-alanine, broth, or carnosine according to the treatments schedule in Table 4. Plasma beta-alanine was below the limit of detection in all subjects on the control treatment. Neither carnosine or anserine were detected in plasma following ingestion of the chicken broth or any of the other treatments. Ingestion of the broth resulted in a peak concentration in plasma of 427.9 (SD 161.8) μM. Administration of carnosine equivalent to 20 milligrams per kilogram body weight of beta-alanine in one test subject resulted in an equivalent increase in the plasma beta-alanine concentration.

Administration of all treatments except control resulted in an increase in the plasma taurine concentration. The changes in taurine concentration mirrored closely those of beta-alanine. Administration of broth, a natural food, caused an equivalent increase in plasma taurine, indicating that the response occurs normally following the ingestion of most meals.

Example 3

The effect of administration of three doses of 10 milligrams per kilogram body weight of beta-alanine per day (i.e., administered in the morning, noon, and at night) for seven days on the plasma concentration profiles of beta-alanine and taurine were investigated. The plasma concentration profiles following administration of 10 milligrams per kilogram body weight of beta-alanine were studied in three subjects at the start and end of a seven-day period during which they were given three doses of the beta-alanine per day.

Three male subjects of normal health, aged between 33-53 years were studied. Test subjects received three doses per day of 10 milligrams per kilogram body weight of beta-alanine for eight days. In two subjects, this was followed by a further 7 days (days 9-15) when three doses of 20 milligrams per kilogram body weight per day were given. Subjects reported at 8 am to the blood collection laboratory on days 1 (prior to any treatment given), 8 and 15 following an overnight fast. Subjects were asked not to consume any meat containing meal during the 12 hours preceding the study. On each of these three test days subjects were catheterized and an initial

blood sample taken when the beta-alanine was administered at or close to 9 am, 12 noon, and 3 pm. Blood samples were drawn after 30, 60, 120 and 180 minutes, and analyzed for changes in the plasma concentration of beta-alanine and taurine. 24-hour urine samples were collected over each day of the study and analyzed by HPLC to determine the excretion of beta-alanine and taurine. The treatments are summarized in Table 5.

TABLE 5

Treatment Day beta-alanine	Day 1 10 mg/kg bwt	Day 8 10 mg/kg bwt	Day 15 20 mg/kg bwt
1	+	+	+
2	+	+	+
3	+	+	

The plasma beta-alanine concentrations are summarized in FIG. 9. Each dose resulted in a peak beta-alanine concentration at one-half hour or one hour after ingestion followed by a decline to a 0-10 micromolar basal level at three hours, just prior to administration of the next dose. The response on day 8 of the treatment tended to be less than on day 1, as indicated by the area under the plasma concentration curve.

Example 4

The effect of administration of three doses of 40 milligrams per kilogram body weight of beta-alanine per day (i.e., administered in the morning, noon, and at night) for 2 weeks on the carnosine content of muscle and isometric endurance at 66% of maximal voluntary contraction force was investigated.

Six normal male subjects, aged 25 to 32 years, that did not have evidence of metabolic or muscle disease were recruited into the study. The subjects were questioned regarding their recent dietary and supplementary habits. None of subjects was currently taking supplements containing creatine, or had done so in recent testing supplementation procedures. The physical characteristics of the test subjects are summarized in Table 6.

TABLE 6

Subject	Age (years)	Weight (kg)
1	29	78
2	31	94
3	29	105
4	25	65
5	31	81
6	25	75
7	53	76

Two days before treatment, a preliminary determination of maximal voluntary (isometric) contraction force (MVC) of knee extensors with the subject in the sitting position was carried out. MVC was determined using a Macflex system with subjects motivated by an instantaneous visual display of the force output. For each subject, two trials were carried out to determine endurance at 66% MVC sustained until the target force could no longer be maintained despite vocal encouragement. This first contraction was subsequently followed by a rest period of 60 seconds, with the subject remaining in the isometric chair. After the rest period, a second contraction was sustained to fatigue. Following a second rest of 60 seconds, a third contraction to fatigue was undertaken.

One day before treatment, the subjects reported to the isometric test laboratory between 8 and 10 am. MVC was determined and endurance at 66% MVC over three contractions with 60 second rest intervals, as described above, was determined. Measurements were determined using the subject's dominant leg. A biopsy of the lateral portion of the vastus lateralis was taken again from the dominant leg.

On day 1 of the treatment study, subjects reported to the blood sampling laboratory at 8 am following an overnight fast and a minimum of 12 hours since the last meat containing meal. Following catheterization and a basal blood sample, each subject followed the supplementation and blood sampling protocol described in Example 3. A dose of 10 milligrams per kilogram body weight of beta-alanine was administered at time 0 (9 am), 3 hours, and 6 hours.

On days 2-15, subjects continued to take three doses of 10 milligrams per kilogram body weight of beta-alanine.

In the morning of day 14, post-treatment isometric exercise tests were conducted on the dominant leg to determine MVC and endurance at 66% MVC relative to the 66% MVC measured on the day prior to treatment. In the afternoon, a muscle biopsy was taken of the vastus lateralis from close to the site of the biopsy taken on the day before treatment.

On day 15, the procedures followed on day 1 were repeated to determine any overall shift in the plasma concentration profile of beta-alanine and taurine over the 15 days of supplementation. Mean changes in plasma beta-alanine over 9 hours following the oral ingestion of 10 milligrams per kilogram body weight of beta-alanine at 0, 3 and 6 hours on days 1 and 15 when dosing at 3×10 milligrams per kilogram body weight per day are shown in FIG. 10.

One additional test subject (number 7) followed the study, taking three doses 10 milligrams per kilogram body weight for 7 days followed by three doses of 20 milligrams per kilogram body weight for 7 days. No muscle biopsies were taken from this test subject.

There was no apparent change in the muscle carnosine content in the muscle of the six subjects biopsied. Changes in plasma taurine concentrations in the six subjects mirrored those of beta-alanine, as noted in Example 2.

Values from the MVC and endurance at 66% MVC measurements one day before treatment and after 14 days after treatment with three doses of 10 milligrams per kilogram body weight of beta-alanine are listed in Table 7. The mean endurance time at 66% MVC increased in 5 of the 6 subjects. An increase was also seen in subject 7 who had taken the higher dose.

TABLE 7

Subject	MVC 1st try N	MVC 2nd try N	time @ 66% MVC 1st seconds	time @ 66% MVC 2nd seconds	time @ 66% MVC 3rd seconds	Total Contraction Time seconds
Pre						
1	784.5	821.9	48.53	29.03	23.78	100.83
2	814.4	886.2	48.40	26.03	16.90	91.33
3	984.9	970.4	38.15	26.03	16.78	80.95
4	714.6	740.4	89.03	56.15	45.65	190.83
5	1204.8	1217.2	37.65	27.64	21.53	86.83
6	722.4	716.8	46.78	29.40	21.90	98.08
Pre mean	870.9	892.1	51.4	32.4	24.3	108.1
Pre SD	190.6	184.6	19.1	11.7	10.8	41.2
Post						
1	895.6	908.0	47.08	30.38	24.03	101.48
2	832.2	908.0	46.65	31.28	18.40	96.33
3	973.7	952.2	42.65	25.03	16.03	83.70
4	814.1	863.9	114.40	64.28	48.53	227.20

TABLE 7-continued

Subject	MVC 1st try N	MVC 2nd try N	time @ 66% MVC 1st seconds	time @ 66% MVC 2nd seconds	time @ 66% MVC 3rd seconds	Total Contraction Time seconds
5	1246.6	1233.0	42.03	22.78	19.40	84.20
6	760.8	773.3	52.28	31.53	25.95	109.73
Post mean	920.5	939.7	57.5	34.2	25.4	117.1
Post SD	175.7	156.0	28.1	15.2	11.9	54.9
Subject 7						
Pre	858.18	861.54	54.0			
Post	792.54	851.41	62.0			

Example 5

The effect of 4 weeks of beta-alanine supplementation using two dosing regimens and an isomolar dose of L-carnosine (beta-alanylhistidine) administered over 4 weeks on the muscle carnosine content were investigated.

Fifteen male subjects, aged 20 to 29 years with no obvious signs of clinical disease and with heights and weights within the normal range, were recruited into the study (Table 8). All subjects participated in one or more sports and all ate a mixed diet containing variable amounts of meat. A record of each subject's approximate intake of meat during the course of the investigation was made.

TABLE 8

Summary of subjects' physical characteristics for each of the three treatment groups:

Treatment	AGE Mean ± SD	HEIGHT Mean ± SD	MASS Mean ± SD
1	24.4 ± 2.7	182.3 ± 7.5	80.0 ± 15.9
2	23.8 ± 1.9	180.9 ± 5.4	80.6 ± 8.6
3	24.0 ± 3.8	180.1 ± 3.8	80.4 ± 12.1

Five subjects were allocated to one of three treatment groups (1, 2, and 3). During the study, their diet was supplemented with either beta-alanine or carnosine as described in Table 9 (FIG. 17). The supplements were provided in soft gelatine capsules containing either 400 mg beta alanine or 500 mg carnosine.

In Group 1, beta-alanine was administered in 4 separate doses throughout the day (qid) at a steady rate for four weeks.

In Group 2, beta-alanine was administered as 8 separate doses throughout the day, rather than as 4 doses, in an attempt to maintain a more even increase in the blood-plasma concentration. In addition, the dose was increased progressively each week by 800 mg per day.

In Group 3, carnosine was administered at approximately the same isomolar dose as in Group 2, again divided into 8 doses. This treatment, therefore, contained approximately the same amount of beta-alanine as in Group 2, when hydrolyzed to its constituent amino acids.

The subjects took the supplements at the times indicated in Table 9 (FIG. 17). A single muscle biopsy of the vastus lateralis was taken before and at the end of the supplementation using the percutaneous needle biopsy procedure of Bergström (1962). In brief, the procedure involves the insertion of a hollow bored needle under local anesthetic and sterile conditions to obtain specimens around 20-40 mg containing approximately 100-700 muscle fibers. The skin and subcutaneous tissue is anesthetized with 1% lignocaine (avoiding contact with the muscle). An incision is made to the skin and deep fascia with a scalpel blade. The needle minus central rod is inserted into the muscle. The muscle bulk is pressed into a needle side-window. A sample is cut by ramming an inner, sharpened cylinder along the needle. The needle is removed and the central rod is used to evacuate the specimen. The wound is then closed.

Muscle samples from the subjects were frozen in liquid nitrogen, freeze-dried and analyzed for muscle carnosine and taurine contents by HPLC. Table 9 (FIG. 17) shows a breakdown of the dosing strategies employed in each of the three treatment groups. Results: There was no change in body mass in either beta-alanine or carnosine supplemented subjects.

Changes in Muscle Carnosine (Table 10 and FIGS. 12 and 13).

A significant increase in muscle carnosine content was recorded for the subjects in Groups 1 and 3. In Group 2, one subject (no. 10) with the highest initial carnosine content (initial carnosine content: 33.3 mmol/kg dry muscle) showed no change in his muscle carnosine content (post content: 33.7 mmol/kg dm). When this subject was deleted from Group 2, this group showed a significant increase of the same order as seen in the other Groups. Subject 10 was a medium to high consumer of meat and otherwise unremarkable.

Supplementation with either beta-alanine or carnosine at the same dose (Groups 2 and 3) appeared to be equally effective in increasing the muscle carnosine content.

The pattern of change is reminiscent of the changes observed with creatine loading and may suggest that there is a threshold which is quickly reached, with further supplementation having no further effect. In the case of subject 10, while not wishing to be bound by this theory, a threshold appears to have been reached even before the start of supplementation. However, there are exceptions to the notion of an upper threshold, notably subjects 6 (post supplementation camosine: 45.9 mmol/kg dry muscle) and 15 (post supplementation carnosine: 68.9 mmol/kg dm). Subjects 6 and 15 were unremarkable in either their dietary patterns or participation in physical exercise.

Table 10 is a summary of data for carnosine muscle concentrations for treatment Groups 1 to 3. Treatment Group 2, in italics, is without subject 10 who did not exhibit an increase in muscle carnosine concentration. The initial carnosine concentration in subject 10 was the highest of all subjects and may have already been at an "upper threshold" level prior to supplementation.

TABLE 10

	Treatment			
	1 n = 5	2 n = 5	*2 n = 4*	3 n = 5
Mean pre	19.58	24.23	*21.96*	23.15
SD pre	3.71	5.27	*1.64*	5.07
Mean post	27.38	35.27	*35.67*	39.52
SD post	2.96	6.18	*7.08*	16.95
Mean difference	7.80	11.04	*13.72*	16.37
SD difference	0.81	9.20	*8.08*	12.06
Sign	***	ns	*	*
Min difference	6.99	0.35	*8.62*	7.05
Max difference	9.08	25.77	*25.77*	37.39
Mean % change	42.1	51.6	*64.2*	65.8
SD % change	14.9	46.5	*42.7*	31.8
Min % change	31.5	1.1	*41.0*	38.2
Max % change	68.0	128.2	*128.2*	118.7

Changes in Muscle Beta-Alanine and Histidine (Table 11 and FIG. 14)

The muscle beta-alanine concentration was below the limit of detection (<0.2 mmol/kg dm) before and at the end of supplementation. In some subjects, a final dose of beta-alanine was taken within 1 to 2 hours of the final muscle biopsy.

There was no change in the muscle histidine concentration with supplementation with either beta-alanine or carnosine, the latter having the potential to release histidine into the general circulation. There was no decrease in the histidine concentration in response to the increased synthesis of carnosine (each mole requiring one mole of histidine).

Changes in Muscle Taurine (Table 12 and FIG. 15 and FIG. 16)

While beta-alanine at high concentrations may interfere with the uptake of taurine into tissues, previous observations show an increase in the plasma taurine concentration and loss of taurine in urine following both beta-alanine and carnosine administration, no loss of muscle taurine was noted in this study in any of the three Groups. Marked changes in the muscle taurine content occurred in some individuals, but both increases and decreases were observed. FIG. 15 is a graph illustrating data showing muscle concentration (mean±SD) of taurine before and post supplementation in four different treatment groups. FIG. 16 is a graph illustrating data showing muscle concentration (mean±SD) of taurine before and post supplementation in different subjects.

CONCLUSIONS

These studies demonstrate that the supplements of beta-alanine and carnosine of the invention have the potential to increase the muscle carnosine content. Based on the test results, they appear to be equally effective in increasing carnosine in tissue.

The changes in the muscle buffering capacity help maintain the intracellular microenvironment during intense exercise, countering the accumulation of H^+. As such, supplementation with beta-alanine or compounds delivering beta-alanine on ingestion may have a positive effect on exercise capacity in sports and those general daily activities leading to lactate accumulation. In view of the other chemical activities ascribed to carnosine (as an anti-oxidant and anti-glycating agent), an increase in carnosine concentration may have other beneficial effects apart from those arising from an increase in muscle buffering capacity.

Four weeks of supplementation did not result in any apparent loss of taurine in the muscles.

TABLE 11

summary data for histidine muscle concentrations in treatment Grou s 1 to3: p

	Treatment 1 n = 5	2 n = 5	3 n = 5
mean pre	5.76	5.56	7.01
SD pre	0.59	0.63	2.60
mean post	5.12	5.51	5.38
SD post	1.17	0.87	1.39
mean diff	−0.64	−0.05	−1.63
SD diff	1.24	0.70	2.87
Sign	ns	ns	ns
% change	−10.59	−0.78	−17.47
SD % change	21.65	12.65	29.56

TABLE 12

Summary data for taurine muscle concentrations in treatments Grou s 1 to3: p

	Treatment 1 n = 5	2 n = 5	3 n = 5
Mean pre	36.52	28.68	35.40
SD pre	7.77	5.29	8.92
Mean post	33.70	27.54	32.32
SD post	7.98	8.77	15.19
Mean difference	−2.82	−1.15	−3.08
SD difference	7.96	6.04	13.61
Sign	ns	ns	ns
% change	−6.28	−4.46	−7.66
SD % change	21.54	24.06	34.97

A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.

What is claimed is:

1. A human dietary supplement, comprising a beta-alanine in a unit dosage of between about 0.4 grams to 16 grams, wherein the supplement provides a unit dosage of beta-alanine.

2. The human dietary supplement of claim 1, further comprising a creatine in a unit dosage of between about 0.4 grams to 16 grams.

3. The human dietary supplement of claim 1, further comprising an L-histidine in a unit dosage of between about 0.08 grams to 8 grams.

4. The human dietary supplement of claim 2, wherein the composition is formulated for oral, enteral, or parenteral administration.

5. A composition for increasing beta-alanylhistidine dipeptide in a subject, comprising a mixture of creatine and anserine or balenine in an amount for increasing beta-alanylhistidine dipeptide in a subject.

6. The composition of claim 5, further comprising a simple carbohydrate.

7. The composition of claim 6, wherein the simple carbohydrate comprises a glucose.

8. The composition of claim 7, further comprising an insulin.

9. A dietary supplement, comprising a mixture of creatine and anserine or balenine.

10. The dietary supplement of claim 9, further comprising a simple carbohydrate.

11. The dietary supplement of claim 10, wherein the simple carbohydrate comprises a glucose.

12. The dietary supplement of claim 10, further comprising an insulin.

13. A method of regulating hydronium ion concentration in a tissue of a subject, comprising administration of a composition comprising a mixture of creatine and anserine or balenine to the subject to increase beta-alanylhistidine dipeptide synthesis in a tissue, whereby the anaerobic working capacity of the tissue is increased.

14. The method of claim 13, wherein administration is oral, enteral, or parenteral.

15. The human dietary supplement of claim 2, further comprising an L-histidine in a unit dosage of between about 0.08 grams to 8 grams.

16. The method of claim 13, wherein the composition is administered in multiple doses.

17. The method of claim 16, wherein the composition is administered at least two times to eight times in a 24-hour period.

18. The method of claim 13, wherein the total dosage of anserine or balenine over a 24-hour period is at least 0.5 grams.

* * * * *

EXHIBIT H

Docket No.: 028284.0105N2N1
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Roger Harris et al.

Continuation of Application No.: 12/806,356

Filed: August 10, 2010

For: METHODS AND COMPOSITIONS FOR INCREASING THE ANAEROBIC WORKING CAPACITY IN TISSUES

Confirmation No.: Not Yet Assigned

Art Unit: Not Yet Assigned

Examiner: Not Yet Assigned

FIRST PRELIMINARY AMENDMENT UNDER 37 C.F.R. 1.115

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

INTRODUCTORY COMMENTS

Prior to examination on the merits, please amend the above-identified U.S. patent application as follows:

Amendments to the Specification begin on page 2 of this paper.

Remarks/Arguments begin on page 4 of this paper.

AMENDMENTS TO THE SPECIFICATION

Please amend the specification as follows:

Page 1, related applications replace entire heading and paragraph with the following heading and paragraph:

"CROSS-REFERENCE TO RELATED APPLICATIONS"

This application is a continuation of U.S. patent application Ser. No. 12/806,356 which is a continuation of U.S. patent application Ser. No. 12/231,240, filed Aug. 29, 2008, now U.S. Pat. No. 7,825,084, which is a continuation of U.S. patent application Ser. No. 10/717,217, filed Nov. 18, 2003, now U.S. Pat. No. 7,504,376, which claims the benefit of priority under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 60/462,238 filed Apr. 10, 2003 and that is a continuation-in-part (CIP) of U.S. application Ser. No. 10/209,169, filed Jul. 30, 2002, now U.S. Pat. No. 6,680,294, which is a continuation of U.S. application Ser. No. 09/757,782, filed Jan. 9, 2001, now U.S. Pat. No. 6,426,361, which is a continuation of U.S. application Ser. No. 09/318,530, filed May 25, 1999, now U.S. Pat. No. 6,172,098, which is a divisional of U.S. application Ser. No. 08/909,513, filed Aug. 12, 1997, now U.S. Pat. No. 5,965,596, which claims the benefit of foreign priority under 35 U.S.C. § 119 to United Kingdom Application Nos. 9621914.2, filed Oct. 21, 1996, and 9616910.7, filed Aug. 12, 1996. The aforementioned applications are incorporated by reference in their entirety.

~~RELATED APPLICATIONS~~

~~This application is a continuation of U.S. patent application Ser. No. 12/231,240, filed Aug. 29, 2008, which is a continuation of U.S. patent application Ser. No. 10/717,217, filed Nov. 18,~~

~~2003, now U.S. Pat. No. 7,504,376, which claims the benefit of priority under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 60/462,238 filed Apr. 10, 2003 and that is a continuation in-part (CIP) of U.S. application Ser. No. 10/209,169, filed Jul. 30, 2002, now U.S. Pat. No. 6,680,294, which is a continuation of U.S. application Ser. No. 09/757,782, filed Jan. 9, 2001, now U.S. Pat. No. 6,426,361, which is a continuation of U.S. application Ser. No. 09/318,530, filed May 25, 1999, now U.S. Pat. No. 6,172,098, which is a divisional of U.S. application Ser. No. 08/909,513, filed Aug. 12, 1997, now U.S. Pat. No. 5,965,596, which claims the benefit of foreign priority under 35 U.S.C. § 119 to United Kingdom Application Nos. 9621914.2, filed Oct. 21, 1996, and 9616910.7, filed Aug. 12, 1996. The aforementioned applications are incorporated by reference in their entirety.~~

REMARKS

By this Preliminary Amendment, Applicants have incorporated the priority information to the continuation application filed concurrently. No new matter is being introduced by this amendment. Thus, Applicants request entry of this Amendment.

Comments in Support of the Claims

The claims encompass human dietary supplements that contain the individual amino acid beta-alanine, an ester of the individual amino acid beta-alanine, or an amide of the individual amino acid beta-alanine. The claims do not encompass human dietary supplements in which the beta-alanine is a component of a dipeptide, oligopeptide, or polypeptide. Consequently, a supplement containing carnosine alone, without any free beta-alanine amino acid as currently claimed, would not be within the scope of the claim, while supplements containing carnosine and the free beta-alanine would be within the claimed scope. The claims are also directed to human dietary supplements that delay the onset of fatigue. This is supported by the specification, for example at Col. 3, ll.50-65 of U.S. Patent No. 6,426,361, to which the present application claims priority.

The claims also encompass human dietary supplements that protect the function of the creatine-phosphorylcreatine system in the human body and assist in the production of energy in the human body, which are supported by the specification at, for example Col. 1, ll. 40-65 of U.S. Patent No. 6,426,361.

The claims also make it clear that the claims do not encompass human dietary supplements in which the beta-alanine is a component of a dipeptide, oligopeptide, or polypeptide, which is contrary to the construction of the court in NAII *et al.* v. VPX *et al.* C.A.

No. 09-626-GMS, Claim Construction Order, dated May 31, 2011 (cited in the Information Disclosure Statement submitted herewith). Despite the disclosure in the specification that beta-alanine can be a component of a dipeptide, oligopeptide, or polypeptide, applicants hereby clearly disavow any claim scope in the instant claims that encompasses beta-alanine as a component of a dipeptide, oligopeptide, or polypeptide.

Furthermore, the claims encompass human dietary supplements. By human dietary supplements the applicants mean an addition to the human diet in a pill, capsule, tablet, powder, or liquid form, which is not a natural or conventional food, and which effectively increases the function of tissues when consumed. This is supported by the specification at Col. 1, ll. 18-25; Col. 3, ll. 54-59 and Examples 1-4 of U.S. Patent No. 6,426,361, for example. To be clear, the term "human dietary supplement", as claimed, does not encompass, and does not mean, a natural or conventional food, such as chicken or chicken broth, for example.

These claims also ensure that the claimed subject matter is not encompassed by any alleged prior art and/or arguments set forth in Defendant Vital Pharmaceutical's First Supplemental Response to Plaintiff's Interrogatory No. 12, served April 19, 2011 ("Interrogatory Response") (submitted herewith in the Information Disclosure Statement). Specifically, the claims do not encompass beta-alanine as a component of a dipeptide, oligopeptide, or polypeptide, as disclosed by the Setra reference (cited in the Information Disclosure Statement submitted herewith). Additionally, the use of beta-alanine in a human dietary supplement is not disclosed by "Carnosine Metabolism and Function in the Thoroughbred Horse," of Dunnet M. (1996) ("The Dunnet Thesis") (cited in the Information Disclosure Statement submitted herewith). Furthermore, the claims do not encompass beta-alanine as a component of a

dipeptide, oligopeptide, or polypeptide and therefore, the claims are not anticipated by beef, pork, chicken, meat extract supplements and predigested meat/protein supplements, as was alleged in the above-referenced court proceedings. To the extent these compositions contain the individual amino acid beta-alanine, or an ester or amide of the individual amino acid beta-alanine, applicants respectfully submit that the definition of human dietary supplement set forth above does not encompass compositions such as those set forth by Defendant in the Interrogatory Response (referred to above and cited in the Information Disclosure Statement submitted herewith). Moreover, because the term "human dietary supplement," as defined above, is not a natural or conventional food, the claims do not encompass naturally occurring compositions and are patentable under 35 U.S.C. § 101.

Applicant believes no fee is due with this response. However, if a fee is due, please charge our Deposit Account No. 50-2228, under Order No. 028284.0105N2N1 from which the undersigned is authorized to draw.

Dated: August 22, 2011

Respectfully submitted,

By____________________

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Registration No.: 60,464
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EXHIBIT I

Case 1:09-cv-00626-GMS Document 125 Filed 05/31/11 Page 1 of 4 PageID #: 2168

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

NATURAL ALTERNATIVES INTERNATIONAL, INC., *et al.*,

Plaintiffs,

v.

VITAL PHARMACEUTICALS, INC., and DNP INTERNATIONAL CO., INC.,

Defendants.

C.A. No. 09-626 (GMS)

VITAL PHARMACEUTICALS, INC.,

Counterclaim/Third-Party Plaintiff,

v.

NATURAL ALTERNATIVES INTERNATIONAL, INC., and COMPOUND SOLUTIONS, INC.,

Counterclaim/Third-Party Defendants.

COMPOUND SOLUTIONS, INC.,

Third-Party Plaintiff,

v.

DNP INTERNATIONAL CO., INC.,

Defendant.

ORDER CONSTRUING THE TERMS OF U.S. PATENT NOS. 5,965,596; 6,172,098; AND 6,426,361

After having considered the submissions of the parties and hearing oral argument on the matter, IT IS HEREBY ORDERED, ADJUDGED, and DECREED that, as used in the asserted claims of U.S. Patent Nos. 5,965,596 (the "'596 patent"); 6,172,098 (the "'098 patent"); and 6,426,361 (the "'361 patent"):[1]

1. The term "dietary supplement" will not be construed because it is not a limitation.[2]
2. The term "beta-alanine" is construed to mean "beta-alanine in the form of the individual amino acid, or as a component of a dipeptide, an oligopeptide, or a polypeptide, or an active derivative thereof."[3]

---

[1] The three patents-in-suit are from the same patent family and appear to have the same specification. (See D.I. 74 at 2, n. 2.) Therefore, the court will use the singular form of specification and refer to the '361 patent specification.
[2] A threshold issue is whether a term located in a claim's preamble is a limitation that must be construed. "In general, a preamble limits the invention if it recites essential structure or steps, or if it is 'necessary to give life, meaning, and vitality' to the claim. Conversely, a preamble is not limiting 'where a patentee defines a structurally complete invention in the claim body and uses the preamble only to state a purpose or intended use for the invention.'" *Catalina Mktg. Int'l v. Coolsavings.com, Inc.*, 289 F.3d 801, 808 (Fed. Cir. 2002) (internal citations omitted). Here, the court finds that that the term "dietary supplement," which only appears in the preamble of various claims, is not a limitation requiring construction. Interestingly, this term appears in the preambles of claims in which the body contains the same exact language as the body of claims that have "composition" in the preamble. (See, e.g., '361 patent, claims 1and 5; claims 10 and 17; and claims 22 and 27.) At first glance this may make it seem like the term must be a limitation. On closer examination, however, the principles articulated by the Federal Circuit are not satisfied. In particular, there is no evidence that the patentees used this term to distinguish prior art. *See Catalina Mktg. Int'l*, 289 F.3d at 808 ("[C]lear reliance on the preamble during prosecution to distinguish the claimed invention from the prior art transforms the preamble into a claim limitation because such reliance indicates use of the preamble to define, in part, the claimed invention."). Also, the specification does not demonstrate that the term was a necessary and defining aspect of the invention. *See On Demand Mach. Corp. v. Ingram Indus.*, 442 F.3d 1331, 1343 (Fed. Cir. 2006) ("In considering whether a preamble limits a claim, the preamble is analyzed to ascertain whether it states a necessary and defining aspect of the invention, or is simply an introduction to the general field of the claim."). In fact, the specification simply notes that the claimed "composition *can be* a dietary supplement." ('361 patent, col. 3, l. 41 (emphasis added).) For these reasons, the court agrees with the defendants that the term "dietary supplement" is not a claim limitation requiring construction.
[3] Here, the patentees acted as their own lexicographer in defining the disputed term and that lexicography governs. *See Phillips v. AWH Corp.*, 415 F.3d 1303, 1316 (Fed. Cir. 2005). Contrary to the plaintiffs' contentions, given the unambiguous nature of the patentees' definition, the court need not apply the analysis set forth in the *Genentech* case. *But see Genentech Inc. v. Wellcome Found.*, 29 F.3d 1555, 1563-64 (Fed. Cir. 1994) ("These diverse definitions reflect either inartful drafting, a conscious attempt to create ambiguity about the scope of the claims, or a desire to claim a wide variety of materials not described or enabled in the specification."). Moreover, the court agrees with the defendants that, based upon an examination of the prosecution history as a whole, prosecution disclaimer has not been established in this case. *See Storage Tech. Corp. v. Cisco Sys.*, 329 F.3d 823, 833-34 (Fed. Cir. 2003). Thus, the court adopts the defendants' proposed construction, except for the parenthetical "such as carnosine." The court could not find a compelling reason to inject (no pun intended) the parenthetical into a definition clearly established by the patentees. (See '361 patent, col. 2, ll. 42-46.) Furthermore, as indicated in the specification, and well known to one of ordinary skill in the art, dipeptides include carnosine. (See '361 patent, col. 2, ll. 1-2.)

3. The term "L-histidine" is construed to mean "L-histidine in the form of the individual amino acid, or as a component of a dipeptide, an oligopeptide, or a polypeptide, or an active derivative thereof."[4]

4. The term "unit dosage form" is construed to mean "doses of a certain serving size that can be taken all at once, or in multiple parts throughout the day."[5]

5. The term "providing an amount of [beta-alanine or L-histidine] to blood or blood plasma effective to increase beta-alanylhistidine dipeptide synthesis in a human tissue" is construed to have its plain and ordinary meaning.[6]

6. The term "increasing a concentration of insulin in the blood or blood plasma" is construed to have its plain and ordinary meaning.[7]

7. The term "active derivative" is construed to mean "a compound derived from, or a precursor of, the substance that performs in the same or similar way in the body as the substance, or which is processed into the substance and placed into the body."[8]

---

[4] See footnote 3.

[5] The court rejects the defendants' construction because there is no intrinsic support for it. The intrinsic record, however, does support the plaintiffs' construction. See, e.g., '361 patent, col. 3, ll. 46-49; col. 5, l. 56 - col. 6, l. 3; and col. 6, ll. 6-10.

[6] The court rejects the plaintiffs' construction because it appears to impose additional limitations. As the defendants' emphasized at the *Markman* hearing, "there is no limitation on the way that the providing step occurs." (Tr. at 102:12-13.) Indeed, the specification merely states that the "providing steps of the methods can include ingestion or infusion (e.g., injection) of a composition . . . or a combination of ingestion and infusion." ('361 patent, col. 3, ll. 10-13.) There is no indication in this disputed claim language that the providing step is so limited. In fact, to the contrary, the patentees explicitly specified when the providing step includes "ingestion of a composition" ('596 patent, claim 7; '098 patent, claim 7) or "infusion of a composition" ('596 patent, claim 8; '098 patent, claim 8). "Ultimately, the interpretation to be given a term can only be determined and confirmed with a full understanding of what the inventors actually invented and intended to envelop with the claim. The construction that stays true to the claim language and most naturally aligns with the patent's description of the invention will be, in the end, the correct construction." *Phillips*, 415 F.3d at 1316.

[7] Again, the court rejects the plaintiffs' construction, which appears to impose additional limitations. The specification states that the "methods can include increasing a concentration of insulin in the blood or blood plasma. The concentration of the insulin can be increased, for example, by injection of insulin." ('361 patent, col. 3, ll. 14-16.) The disputed claim term, however, is not expressly limited. The court is persuaded by the defendants' argument that it should not add limitations specifying mechanisms when the claim only addresses effect. (Tr. at 109:24-110:3.)

[8] The court adopts the defendants' construction. The patentees explicitly defined the term "active derivative." (See '361 patent, col. 2, ll. 46-49.) Therefore, the patentees' lexicography governs. *See Phillips*, 415 F.3d at 1316.

8. The term "mixture" is construed to mean "a composition, physical combination, or blend of substances that are not chemically bonded to one another."[9]

Dated: May 31, 2011

CHIEF, UNITED STATES DISTRICT JUDGE

[9] The parties agreed upon this construction prior to the *Markman* hearing as represented in their joint claim chart. (See D.I. 74 at 13.)

EXHIBIT J

Gut, 1970, **11,** 250-254

Intestinal absorption of carnosine and its constituent amino acids in man[1]

A. M. ASATOOR, J. K. BANDOH[2], A. F. LANT, M. D. MILNE, AND F. NAVAB

From the Medical Unit of the Westminster Hospital, London

SUMMARY Serum concentrations of β-alanine and L-histidine are compared in five normal adults after ingestion of the dipeptide carnosine (β-alanyl-L-histidine) and after equivalent amounts of the constituent free amino acids. The results indicate that absorption is significantly more rapid after the ingestion of the amino acids than after the dipeptide. The use of the test in a case of Hartnup disease suggests that carnosine is taken up by intestinal cells as the dipeptide, but subsequent hydrolysis and delivery of the constituent amino acids to the portal blood is a slower process than transport of the free amino acids themselves.

Current views of intestinal amino acid absorption suggest that at least two mechanisms are involved. Free amino acids are actively transported against a concentration gradient from the intestinal lumen to the portal capillaries (Wiseman, 1951) and small peptides are taken up intact from the intestinal lumen, but are hydrolysed by the peptidases of the intestinal mucosal cells and appear in the portal blood or in serosal fluid as the corresponding free amino acids (Wiggans and Johnston, 1959; Newey and Smyth, 1960 and 1962). Evidence supporting intestinal uptake of intact peptides in man has been reported for the di- and tripeptides of glycine (Craft, Geddes, Hyde, Wise, and Matthews, 1968), plasma glycine levels being considerably higher 30 minutes after the ingestion of each peptide than after a corresponding amount of free glycine. This indicated a more rapid cellular uptake of peptide molecules than of the larger number of molecules of free glycine. Because of the marked differences in specific aminopeptidase activities within the microvillous membrane of the intestinal epithelium (Rhodes, Eicholz and Crane, 1967), conclusions drawn from studies with glycyl-glycine may not be generally applicable to the cellular mechanism of absorption of other peptides. Quite apart from the question of species differences in absorptive behaviour, the problem of oligopeptide absorption is complicated by the fact that about 400 different dipeptides can be formed from the amino acids of proteins, and the figure becomes much higher if tri- or higher peptides are considered.

Investigation of peptide absorption by tolerance tests in man involves difficulties in the supply of oligopeptides of sufficient purity, as these compounds are in most cases difficult to prepare in bulk. The dipeptide carnosine (β-alanyl-L-histidine) is readily available, and is necessarily ingested in considerable amounts by all non-vegetarians, as mammalian muscle contains up to 0·6% of carnosine (Davey, 1957). This paper compares serum levels of the constituent amino acids, β-alanine and L-histidine, after ingestion of the peptide and corresponding quantities of the two free amino acids.

Methods

Five normal adults were investigated, each individual ingesting carnosine and equivalent amounts of a mixture of β-alanine and L-histidine after an overnight fast. The two tolerance tests in each subject were carried out at

[1]Address for reprints: Medical Unit, Westminster Hospital, Page Street Wing, London, SW1.
[2]Present address: Military Hospital, Accra, Ghana.

intervals of at least two weeks. Dosage of carnosine was 0·286 m mole/kg body weight, corresponding to 20 m mole per standard 70 kg male. Histidine and β-alanine were taken together in an amount which would be produced after hydrolysis of the above dose of carnosine. Both the dipeptide and the amino acid mixture were taken dissolved in 500 ml water. Blood samples were obtained at zero time, and at 15, 30, and 45 minutes after ingestion of the solution. Serum for analysis was obtained with the minimum of delay. In one subject additional samples were analysed at 60 and 90 minutes after the mixture.

MATERIALS AND CHEMICAL METHODS

Amino acids were analysed on the Technicon amino acid analyser using the standard procedure described in the Technicon Handbook (1966).

Carnosine, β-alanine, and L-histidine were obtained from commercial sources and were found to be chromatographically pure.

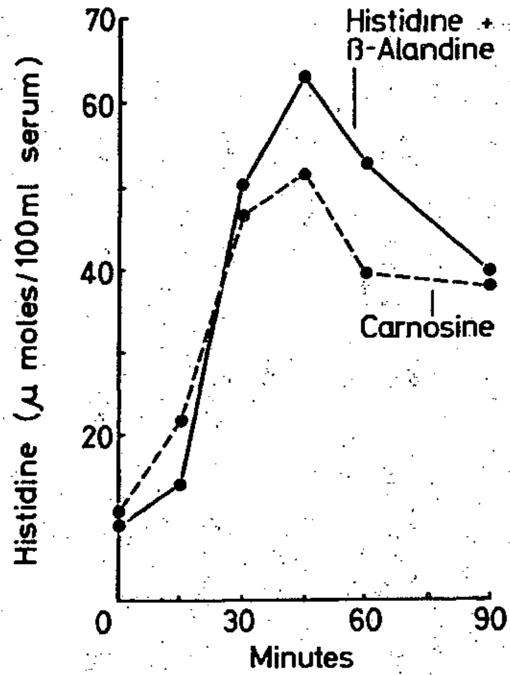

Fig. 1 *Concentrations of serum histidine after ingestion of carnosine and the constituent free amino acids in a normal subject.*

Results

Peripheral paraesthesiae occurred in all subjects from about 15 to 45 minutes after ingestion of carnosine and of the mixture of the two amino acids, but otherwise no abnormal symptoms occurred. Since similar effects have not been described after ingestion of larger quantities of histidine alone, the paraesthesiae were presumably due either to β-alanine or to one of its metabolites. No β-alanine was detected in the basal serum specimens, but histidine concentration averaged 11·2 μ moles/100 ml (range 8·6-17·0). All values for histidine in subsequent serum specimens are recorded as increments above the basal concentration. Carnosine was not detected in any serum sample.

Tolerance curves in the subject in whom additional serum samples were analysed are given in Figures 1 and 2. Peak concentrations of β-alanine occurred in the sample taken after 30 minutes, and of histidine in the 45-minute specimen. In the period of 15 to 30 minutes after the drink the maximum rate of increase in serum amino acid concentration was 1·25 times as rapid for β-alanine as for histidine in both tolerance tests. Similarly, the subsequent rate of decline after the peak concentrations was greater in the case of β-alanine. Comparing the two tolerance curves, the rate of rise of both serum β-alanine and of histidine in the 15 to 30-minute period was 1·45 times as great after the free amino acids than after carnosine.

The individual results of the tolerance tests are given in Table I, and the means of concentrations from the five subjects in Figures 3 and 4.
5 In 19 out of 20 cases increments of serum histidine

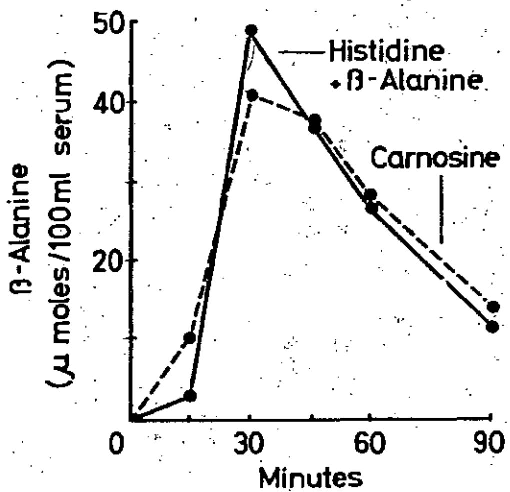

Fig. 2 *Concentrations of serum β-alanine after ingestion of carnosine and the constituent free amino acids in a normal subject.*

and of β-alanine in the same subject at 30 and 45 minutes after the drink were greater after ingestion of the free amino acids than after carnosine. In the remaining analysis the result was identical. This could have occurred by chance alone at a probability value of one in 400 times. As the same individuals were used in both tolerance tests, the method of the paired t test (Snedecor, 1946) is statistically valid. Using this technique the increments of serum amino acids are significantly higher after ingestion of the free amino acids in the 30- and 45-minute samples for histidine and in the 30-minute sample for β-alanine (Table I). In the 45-minute samples for β-alanine, the results are lower after carnosine ingestion, but the difference is not significant ($0{\cdot}1 > P > 0{\cdot}05$). The peak increment of β-alanine occurred at 30 minutes in three of the five subjects, and therefore the 45-minute sample in this case is not a satisfactory representation of the absorption rate of the amino acid. It can be concluded that absorption of both β-alanine and of histidine is significantly more rapid after ingestion of the free amino acids than after ingestion of the equivalent amount of carnosine.

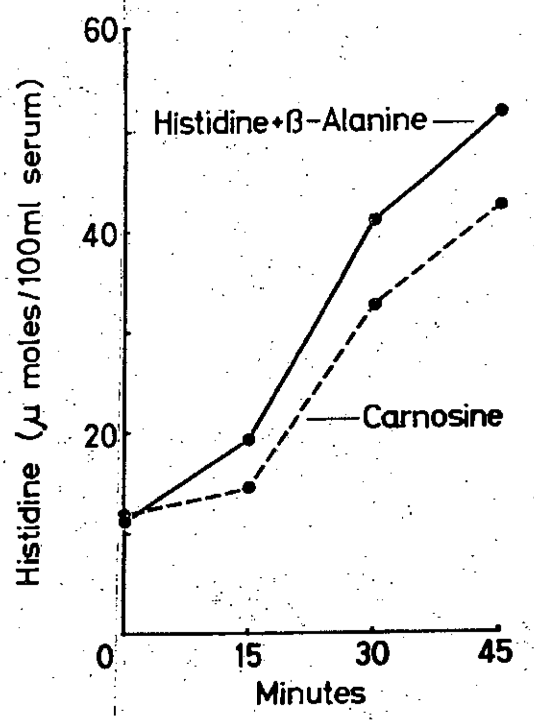

Fig. 3 *Mean serum concentrations of histidine during a 45-minute period after ingestion of carnosine and the constituent free amino acids in five normal subjects.*

	Subject	Free Amino Acids (X_1)	Carnosine (X_2)	($X_1 - X_2$)	Deviation ($X_1 - X_2 - \bar{x}$)	Squared Deviations
Histidine at 30 minutes	1	41·0	36·4	4·6	−3·7	13·69
	2	36·2	24·2	12·0	3·7	13·69
	3	51·2	41·1	10·1	1·8	3·24
	4	17·2	5·0	12·2	3·5	12·25
	5	4·2	1·7	2·5	−5·8	33·64
	Total	149·8	108·4	41·4		76·51
	Mean	30·0	21·7	$\bar{x} = 8{\cdot}3$		$S^2 = 19{\cdot}13$
		$t = 4{\cdot}30$		$P < 0{\cdot}02$		
Histidine at 45 minutes	1	54·0	41·2	12·8	2·9	8·41
	2	41·0	36·0	5·0	−4·9	24·01
	3	48·8	48·7	0·1	−9·8	96·04
	4	35·4	14·4	21·0	11·1	123·21
	5	26·8	16·1	10·7	1·8	3·24
	Total	206·0	156·4	49·6		254·91
	Mean	41·2	31·3	$\bar{x} = 9{\cdot}9$		$S^2 = 63{\cdot}73$
		$t = 2{\cdot}77$		$P < 0{\cdot}05$		
β-alanine at 30 minutes	1	48·4	41·0	7·4	−2·8	7·84
	2	37·4	22·7	14·3	4·1	16·81
	3	55·4	33·6	19·8	9·6	92·16
	4	15·0	5·6	9·4	−0·8	0·64
	5	1·5	1·2	0·3	−9·9	98·01
	Total	157·7	104·1	51·2		215·46
	Mean	35·5	20·8	$\bar{x} = 10{\cdot}2$		$S^2 = 53{\cdot}87$
		$t = 3{\cdot}11$		$P < 0{\cdot}05$		
β-alanine at 45 minutes	1	36·6	36·6	0	−12·2	148·84
	2	30·0	22·4	7·6	−4·6	21·16
	3	48·4	40·4	8·0	−4·2	17·64
	4	39·0	12·0	27·0	14·8	219·04
	5	27·8	9·3	18·5	6·3	39·69
	Total	181·8	120·7	61·1		446·37
	Mean	36·4	24·1	$\bar{x} = 12{\cdot}2$		$S^2 = 111{\cdot}59$
		$t = 2{\cdot}58$		$0{\cdot}1 < P < 0{\cdot}05$		

Table I *Increments in serum histidine and β-alanine after ingestion of carnosine and free constituent amino acids (μmole/100 ml)*

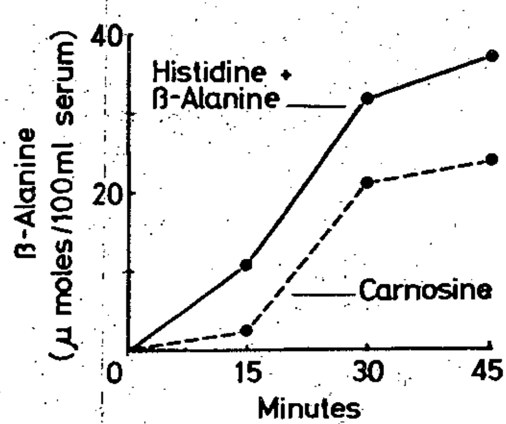

Fig. 4 *Mean serum concentrations of β-alanine during a 45-minute period after ingestion of carnosine and the constituent free amino acids in five normal subjects.*

Subject	*Serum Histidine (μ moles/100 ml/min)*		*Serum β-analine (μ moles/100 ml/min)*	
	After Free Amino Acids	*After Carnosine*	*After Free Amino Acids*	*After Carnosine*
1	2·43	1·65	3·01	2·06
2	1·18	1·52	1·02	1·43
3	2·08	2·56	2·04	2·22
4	1·12	0·34	1·00	0·37
5	1·51	0·96	1·76	0·54
Mean normal	1·66 ± 0·57	1·41 ± 0·83	1·77 ± 0·83	1·32 ± 0·85
Hartnup disease	0·10	1·58	2·50	1·83

Table II *Maximum rates of increment of serum histidine and β-alanine after ingestion of carnosine and the constituent free amino acids in five normal subjects and a case of Hartnup disease*

Discussion

Comparisons of the absorption of amino acids after ingestion of carnosine and of the equivalent amounts of free β-alanine and histidine in this investigation are the opposite of those obtained by Craft *et al* (1968), comparing the rates of absorption of free glycine and of the peptides glycyl-glycine and glycyl-glycyl-glycine. We have confirmed the latter results using the more exact method of ion-exchange chromatography (Technicon Handbook, 1966). In addition, urinary excretion of free glycine in the 45-minute period after ingestion of glycyl-glycine is approximately twice as high as that after ingestion of the equivalent amount of free glycine, a result to be expected when, after ingestion of the peptide, serum levels of the amino acid are consistently higher during this period.

A superficial and obvious interpretation of the conflicting results would be that there is a fundamental difference between the intestinal absorption of the glycine peptides and that of carnosine. The glycine peptides are presumably taken up intact from the gut lumen and subsequently undergo hydrolysis with delivery of free glycine into the portal capillary blood. The whole process is more rapid than that involved in the corresponding absorption of twice or three times the number of free glycine molecules. By contrast, the present results might apparently favour the view that carnosine is hydrolysed in the fluid within the intestinal lumen and the derived amino acids are subsequently absorbed, a process obviously less rapid than absorption of equivalent amounts of the free amino acids not necessitating prior hydrolysis. A repetition of the two tolerance curves in a case of Hartnup disease to be reported in detail elsewhere (Navab and Asatoor, 1970) has, however, shown that this interpretation is almost certainly incorrect. The current view of the disordered physiology of Hartnup disease is one of defective transport of many mono-amino mono-carboxylic amino acids both in the proximal renal tubular cells and in the jejunal epithelium (Milne, Crawford, Girão, and Loughridge, 1960; Jepson, 1966). Although not absolutely proven for every single involved amino acid, all the amino acids excreted at abnormally high clearance by the kidney are probably poorly absorbed in the jejunum. Clearances of histidine in Hartnup disease are at the level of the glomerular filtration rate, and are in fact higher than those of any other of the involved amino acids (Evered, 1956). By contrast, β-alanine, a member of a separate amino acid transport group (Scriver, Pueschel, and Davies, 1966; de la Noüe, Newey, and Smyth, 1969), is not excreted in excess in Hartnup disease. Jejunal absorption of β-alanine is, therefore, likely to be normal, and that of L-histidine to be defective in Hartnup disease. Table II gives the maximum rate of serum amino acid increment, during the absorptive phase in the case of Hartnup disease and in the normal subjects. β-Alanine absorption is seen to be within normal limits after both carnosine and free amino acid ingestion, whereas histidine absorption in Hartnup disease is normal after carnosine but grossly defective after ingestion of the free amino acid. The result may explain the reason why cases of Hartnup disease preserve an almost normal nutritional status despite gross defects in absorption of many essential amino acids. Thus, tolerance curves for the essential amino acid phenylalanine were almost completely flat in this case of the disease, and absorption of the free amino acid would be completely inadequate to sustain life. Probably this and other essential amino acids are absorbed mainly as oligopeptides in Hartnup disease, whereas both free amino acid and oligopeptide are absorbed in normal subjects.

A possible explanation of the results of this investigation is that carnosine is absorbed as the entire molecule, but that subsequent intracellular hydrolysis is relatively slow, and is in fact the rate-limiting step in the total absorptive process. By contrast, intracellular hydrolysis of the glycine peptides must be more rapid and does not cause a significant delay in the transport rate. Full confirmation of this interpretation obviously depends on more complete knowledge of the enzyme kinetics of human intestinal peptidases hydrolysing glycine peptides and carnosine. Glycyl-glycine dipeptidase is a very specific enzyme, and is activated by Co^{++} or Mn^{++} (Smith, 1951). Carnosinase hydrolyses glycyl-L-histidine, L-alanyl-, L-histidine, β-L-aspartyl-L-histidine, and anserine (β-alanyl-I-methyl-histidine) in addition to carnosine, and requires Zn^{++} or Mn^{++} for activity (Hanson and Smith, 1949; Davis, 1956). Similar investigations using these alternative peptides would be both more difficult and more expensive because of lack of availability of the chemicals in pure and bulk supply. The two peptidases are widely distributed in animal tissues, and glycyl-glycine dipeptidase is present in high concentration in intestinal mucosa. Carnosinase has been detected in considerable amount in the intestinal wall of

the rat (Wood, 1957). Carnosine in the dog is absorbed only after hydrolysis either in the intestinal lumen or in the jejunal cells, but re-synthesis of carnosine occurs in the liver, and carnosine is detectable in the plasma of this species (Elwyn, Parikh, and Shoemaker, 1968). Results obtained in the experimental animal may not necessarily be applicable to man. Probably the main importance of this paper is to draw attention to the uncertainties which face any interpretation of tolerance tests as indices of intestinal absorption in man. If it can be shown, as in the case of glycine peptides, that plasma levels are higher after ingestion of the peptide than after the free amino acid, intestinal absorption of the intact peptide is at least probable. By contrast, the converse result, as in the present investigation, may equally well be interpreted as indicating prior luminal hydrolysis of the peptide, or of absorption of the intact peptide with subsequent slow intracellular hydrolysis. Such results in isolation are, therefore, purely factual and any theoretical interpretation is completely speculative.

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EXHIBIT K

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Description

La présente invention concerne une nouvelle composition utilisable en thérapeutique, et plus particulièrement une association à base d'acide aminé, d'une part, et de vitamines, d'autre part, utilisable en thérapeutique cancérologique.

Divers traitements thérapeutiques du cancer ont été proposés en chirurgie, en radiothérapie et en chimiothérapie.

Ainsi, les exérèses chirurgicales, dont les indications sont tributaires de la localisation et de l'envahissement de la tumeur, peuvent être satisfaisantes sur le plan carcinologique, ou simplement palliatives. Cependant, elles constituent une agression certaine pour l'organisme et peuvent entraîner des mutilations définitives.

La radiothérapie, isolée ou associée à un autre traitement, possède une action indéniable sur certains types de tumeurs, mais son action dépasse toujours le cadre régional de la tumeur, entraînant des séquelles qui dépendent de la localisation. Le retentissement sur l'état général du patient dépend des doses cumulées rendues nécessaires en fonction de la masse traitée.

En chimiothérapie, on utilise par exemple des sels de métaux lourds, des agents d'alkylation tels que le cyclophosphamide ou la méchlorétamine, des antimétabolites tels que des dérivés d'uracile ou de cytosine, certains dérivés de la série des antibiotiques comme la bléomycine ou la daunomycine, ou encore des alcaloïdes tels que la vinblastine et la vincristine. Ces composés peuvent être utilisés, selon les cas, isolément ou en association de deux ou plusieurs. Ils présentent généralement des effets secondaires toxiques qui limitent ou interdisent leur utilisation à doses élevées, et ce d'autant plus si le patient présente des tares associées ou des séquelles de chirurgie mutilante ou de radiothérapie.

Toutes ces thérapeutiques, à des degrés divers, ont pour but d'éliminer le maximum ou la totalité des tumeurs cancéreuses, mais aucune d'elles n'exerce d'action sur le génie évolutif du processus cancéreux.

On connait l'importance des vitamines, hydrosolubles et liposolubles, en physiologie et biochimie nutritionnelles. Par contre, leur utilisation pour le traitement du cancer, isolément ou en combinaison avec d'autres composés, n'a pas été décrite jusqu'à présent.

D'autre part, certains acides aminés sont utilisés en thérapeutique pour le traitement de diverses maladies. Ainsi par exemple on sait que la β-alanine possède des propriétés inhibitrices de la vasodilatation périphérique permettant son administration pour le traitement des bouffées de chaleur de la ménopause. Sa toxicité est faible puisque la dose léthale DL_{50} par voie orale chez la souris et le rat, est supérieure à 10 g/kg, et sa tolérance est généralement satisfaisante. Si l'activité antiallergique et antihistaminique de la β-alanine a été décrite, on ne connait par contre aucune application de ce composé, isolément ou en combinaison, au traitement du cancer.

Il est également admis que la β-alanine agit comme médiateur chimique au niveau du système nerveux central, et pourrait agir au niveau cellulaire par rapport au signal calcium.

On connait quelques compositions contenant divers composants, parmi lesquels certains acides aminés et des vitamines ; par exemple "Unlisted Drug" vol. 28 n° 7 cite une composition utilisable comme supplément nutritionnel, contenant de la pyridoxine, de l'acide citrique, de la taurine, etc. Toutefois, l'utilisation spécifique de la β-alanine associée à des vitamines n'a pas été envisagée.

On a maintenant constaté qu'une composition contenant de la β-alanine en association avec diverses vitamines, présente des propriétés permettant son application en thérapeutique pour le traitement du cancer, tandis qu'aucune activité comparable n'est observée quand on utilise isolément chacun de ces composés.

La présente invention a donc pour objet une nouvelle composition à base de β-alanine et de vitamines, utilisable pour le traitement du cancer.

La nouvelle composition conforme à la présente invention comprend au moins une vitamine et de la β-alanine représentée par la formule générale (I) suivante :

$$H_2N\text{-}(CH_2)_2\text{-}COOH \quad (I)$$

ou un de ses sels d'acide ou de base.

Plus particulièrement, la composition conforme à l'invention comprend la β-alanine répondant à la formule (I) ci-dessus et une ou plusieurs vitamines choisies parmi la vitamine A, la vitamine B_1, la vitamine B_2, la vitamine B_5, la pyridoxine, l'acide ascorbique, le tocophérol, la vitamine D_2, et la vitamine PP.

La β-alanine peut être utilisée isolément ou, le cas échéant, en combinaison avec un ou plusieurs autres acides aminés ; par exemple la β-alanine et la glycine, ou la β-alanine et la taurine, peuvent être combinées.

Outre les vitamines et l'acide aminé indiqués ci-dessus, la composition peut également avantageusement comporter des additifs tels que le glucose, le β-carotène, ou d'autres vitamines telles que la biotine et la vitamine B_{12}.

Suivant une forme préférentielle de réalisation de l'invention, la composition comprend la β-alanine, et plusieurs vitamines choisies parmi celles indiquées ci-dessus, ainsi que du glucose, et éventuellement de la taurine, et/ou de la glycine. Il peut être préférable d'ajouter à l'ensemble de ces constituants du β-carotène, précurseur de la vitamine A.

Le rapport pondéral de l'acide aminé au total des vitamines est compris entre 50/1 et 500/1, et de préférence entre 100/1 et 300/1, et la quantité d'acide aminé administrée par jour est comprise entre 50 g et 200 g pour un traitement d'attaque, et entre 10 et 50 g pour un traitement d'entretien chez l'homme adulte. Bien entendu, la dose administrée est déterminée par le médecin en fonction de l'état du patient. Lorsque du glucose est utilisé, il peut représenter entre 10 et 30% en poids de la dose d'acide aminé pour un traitement d'attaque. La quantité de glucose utilisée est sensiblement la même dans un traitement d'attaque et dans un traitement d'entretien.

L'invention concerne également un procédé pour préparer une composition utile en thérapeutique cancérologique.

Les composants constituant la composition de la présente invention peuvent être administrés en mélange ou séparément, de préférence par voie orale. On peut par exemple mélanger l'acide aminé, les vitamines et, le cas échéant, le glucose et les autres additifs, sous forme d'une poudre que l'on peut conditionner sous diverses formes usuelles, par exemple en sachets, en gélules ou capsules. Il est également possible de conditionner séparément l'acide aminé, d'une part, et les vitamines et les autres additifs éventuels, d'autre part, par exemple sous forme de gélules ou capsules administrables par voie orale, séparément, l'administration de chacune de ces deux parties pouvant être espacée dans le temps de quelques minutes à quelques heures, sans excéder toutefois 4 à 5 heures environ.

La composition conforme à la présente invention peut constituer une thérapeutique d'accompagnement des traitements thérapeutiques usuels, chimiques, chimiothérapiques ou radiothérapiques, dans le cas du traitement du cancer. L'avantage de la thérapeutique conforme à la présente invention réside notamment dans le fait que les taitement connus, qui, comme indiqué précédemment, sont traumatisants, voire toxiques, peuvent désormais être utilisés à des doses plus faibles qui évitent les effets secondaires néfastes.

Les résultats expérimentaux ont montré que la composition suivant l'invention ne détruit pas la cellule cancéreuse, mais inhibe sa division cellulaire. Elle possède la propriété, comme les antimitotiques et la radiothérapie, d'agir sur la carcinomédine, potentialisateur de la multiplication cellulaire tumorale. Il est particulièrement intéressant de noter que ces résultats sont obtenus quand les composants sont administrés simultanément, en mélange, mais aussi lorsque les vitamines, d'une part, et l'acide aminé, d'autre part, sont administrés séparément à un intervalle de temps allant de quelques minutes à 4 à 5 heures environ.

Par contre, aucun effet de destruction sur les cellules cancéreuses n'est observé si on administre uniquement un acide aminé, ou des vitamines, ou si leur rapport pondéral ou les doses administrées sont en dehors des limites précitées.

Les études toxicologiques ont montré que la toxicité de la composition suivant l'invention est nulle aux doses administrées pour le traitement du cancer.

Des exemples de compositions conformes à la présente invention sont donnés ci-après à titre non limitatif.

EXEMPLE 1

On prépare un mélange des composants ci-indiqués ci-dessous :

β-alanine	90	g
taurine	10	g
Glucose	30	g
Vitamine A	60.000	UI
Vitamine B_1	24	mg
Vitamine B_2	18	mg
Vitamine B_5	48	mg
Pyridoxine (chlorhydrate)	24	mg
Acide ascorbique	600	mg
Vitamine D_2	12.000	UI
Tocophérol (acétate)	24	mg
Vitamine PP	120	mg
β-carotène	100	mg

Ces composants sont soigneusement mélangés de manière à obtenir une composition homogène correspondant à la dose administrée en 24 heures pour un traitement d'attaque. L'administration est faite par voie orale en quatre parties égales, toutes les 6 heures, soit par prise volontaire, soit additionnée à la solution de nutrition entérale.

EXEMPLE 2

On procède comme dans l'exemple 1 en utilisant les composants ci-dessous pour préparer une composition destinée à un traitement d'entretien :

β-alanine	30	g
Glucose	10	g
Vitamine A	15.000	UI
Vitamine B_1	7,5	mg
Vitamine B_2	7,5	mg
Vitamine B_5	6,5	mg
Pyridoxine (chlorhydrate)	2,3	mg
Biotine	0,1	mg
Vitamine B_{12}	4,5	mg
Acide ascorbique	112	mg
Vitamine D_2	3.000	UI
Tocophérol (acétate)	7,5	mg
Vitamine PP	37,5	mg
β-carotène	60	mg

On obtient ainsi une quantité de composition correspondant à la dose administrée en 24 heures au cours

d'un traitement d'entretien. La composition ci-dessus est administrée en quatre parties égales, toutes les 6 heures.

EXEMPLE 3

En procédant comme dans l'exemple 1, on prépare un mélange des composants suivants :

β-alanine	20	g
Vitamine A	50.000	UI
Vitamine B_1	20	mg
Vitamine B_5	50	mg
Pyridoxine (chlorhydrate)	20	mg
Acide ascorbique	400	mg
Tocophérol	20	mg
Vitamine PP	100	mg

Le mélange est homogénéisé puis réparti entre 5 sachets identiques pour administration par voie orale. Par ailleurs, on prépare, de manière usuelle, 5 sachets dosés à 4 g de taurine.

La composition est administrée à raison de cinq prises d'un sachet du mélange de vitamines et de β-alanine ci-dessus, espacées de quatre heures, suivies à une heure d'intervalle par cinq prises d'un sachet de taurine. On a ainsi un traitement d'attaque qui est renouvelé pendant une durée qui dépend de l'état clinique du patient.

Revendications

1. Composition pour utilisation comme médicament, notamment utilisable dans le traitement du cancer, caractérisée en ce qu'elle comprend au moins une vitamine et de la β-alanine représentée par la formule générale (I) suivante :

$$H_2N\text{-}(CH_2)_2\text{-}COOH$$

ou un de ses sels d'acide ou de base.

2. Composition selon la revendication 1, caractérisée en ce qu'elle comprend une ou plusieurs vitamines choisies parmi la vitamine A, la vitamine B_1, la vitamine B_2, la vitamine B_5, la pyridoxine, l'acide ascorbique, le tocophérol, la vitamine D_2, et la vitamine PP.

3. Composition selon la revendication 1, caractérisée en ce qu'elle contient en outre de la taurine et/ou de la glycine.

4. Composition selon l'une quelconque des revendications 1 à 3, caractérisée en ce qu'elle comprend en outre au moins un additif choisi parmi le glucose, le β-carotène, la biotine et la vitamine B_{12}.

5. Composition selon l'une quelconque des revendications 1 et 2, caractérisée en ce qu'elle comprend la β-alanine, la taurine et/ou la glycine, au moins une vitamine, et du glucose.

6. Composition selon la revendication 1, caractérisée en ce que le rapport pondéral de l'acide aminé au total des vitamines est compris entre 50/1 et 500/1.

7. Composition selon l'une quelconque des revendications 1 à 6, caractérisée en ce qu'elle est sous forme d'un mélange de ses composants, administrable par voie orale.

8. Procédé de préparation d'une composition pour utilisation comme médicament, notamment dans le traitement du cancer selon l'une quelconque des revendications 1 à 6, caractérisé en ce qu'on mélange la β-alanine, les vitamines, et les autres composants, le cas échéant, sous forme de poudre.

Ansprüche

1. Zusammensetzung für die Verwendung als Arzneimittel, das insbesondere für die Behandlung von Krebs verwendbar ist, dadurch gekennzeichnet, daß sie umfaßt mindestens ein Vitamin und β-Alanin der nachstehend

angegebenen allgemeinen Formel (I) :

$$H_2N\text{-}(CH_2)_2\text{-}COOH$$

oder eines seiner Säure- oder Basensalze.

2. Zusammensetzung nach Anspruch 1, dadurch gekennzeichnet, daß sie umfaßt ein oder mehrere Vitamine, ausgewählt aus der Gruppe Vitamin A, Vitamin B_1, Vitamin B_2, Vitamin B_5, Pyridoxin, Ascorbinsäure, Tocopherol, Vitamin D_2 und Vitamin PP.

3. Zusammensetzung nach Anspruch 1, dadurch gekennzeichnet, daß sie außerdem Taurin und/oder Glycin enthält.

4. Zusammensetzung nach einem der Ansprüche 1 bis 3, dadurch gekennzeichnet, daß sie außerdem mindestens ein Additiv, ausgewählt aus der Gruppe Glucose, β-Karotin, Biotin und Vitamin B_{12}, enthält.

5. Zusammensetzung nach einem der Ansprüche 1 und 2, dadurch gekennzeichnet, daß sie umfaßt β-Alanin, Taurin und/oder Glycin, mindestens ein Vitamin und Glucose.

6. Zusammensetzung nach Anspruch 1, dadurch gekennzeichnet, daß das Gewichtsverhältnis zwischen der Aminosäure und der Gesamtmenge der Vitamine zwischen 50 : 1 und 500 : 1 liegt.

7. Zusammensetzung nach einem der Ansprüche 1 bis 6, dadurch gekennzeichnet, daß sie in Form einer auf oralem Wege verabreichbaren Mischung ihrer Bestandteile vorliegt.

8. Verfahren zur Herstellung einer Zusammensetzung für die Verwendung als Arzneimittel, insbesondere für die Behandlung von Krebs, nach einem der Ansprüche 1 bis 6, dadurch gekennzeichnet, daß man das β-Alanin, die Vitamine und gegebenenfalls die anderen Bestandteile. in Form eines Pulvers miteinander mischt.

Claims

1. A composition to be used as a drug, namely for use in the cancer therapy, characterized in that it comprises at least one vitamin and β-alanine, represented by the following general formula (I) :

$$H_2N\text{-}(CH_2)_2\text{-}COOH$$

or one of its base or acid salts

2. A composition according to claim 1, characterized in that it comprises one or several vitamins selected from the group consisting of vitamin A, vitamin B_1, vitamin B_2, vitamin B_5, pyridoxine, ascorbic acid, tocopherol, vitamin D_2 and vitamin PP.

3. A composition according to claim 1, characterized in that, it further contains taurine and/or glycine.

4. A composition according to any of claims 1 to 3, characterized in that it further comprises at least one additive selected from the group consisting of glucose, β-carotene, biotin and vitamin B_{12}.

5. A composition according to any of claims 1 and 2, characterized in that it comprises β-alanine, taurine and/or glycine, at least one vitamin and glucose.

6. A composition according to claim 1, characterized in that the weight ratio of the aminoacid to the total of vitamins is comprised between 50/1 and 500/.1.

7. A composition according to any of claims 1 to 6, characterized in that it is in the form of a mixture of its components, orally dispensable.

8. A process for preparing a composition to be used as a drug, namely for use in the cancer therapy according to any of claims 1 to 6, characterized in that β-alanine, vitamins and other components, optionally, are mixed together in the form of a powder.

Espacenet

Bibliographic data: EP0280593 (A2) — 1988-08-31

Composition on the basis of an amino-acid and vitamins for use in cancer therapy.

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Abstract of EP0280593 (A2)

The composition comprises at least one vitamin and beta -alanine represented by the following formula (I): H2N-(CH2)2-COOH (I> or one of its salts with an acid or base.

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Notice

Description EP0280593

[0001] The present invention relates to a novel composition for use in therapy, particularly a combination of amino acid based on the one hand, and vitamins, on the other hand, can be used in therapeutic oncology.

[0002] Various therapeutic treatments of cancer have been proposed in surgery, radiotherapy and chemotherapy.

[0003] Thus, surgical resection, whose indications depend on the location and the invasion of the tumor, may be satisfactory in terms of oncologic, or just palliative.
However, they are a certain aggression to the body and can cause permanent mutilation.

[0004] Radiation therapy, alone or combined with another treatment, has an undeniable work on certain types of tumors, but its action always exceeds the regional framework of the tumor, resulting in sequelae that depend on the location.
The impact on the overall condition of the patient depends on the cumulative doses become necessary depending on the treated mass.

[0005] Chemotherapy is used for example, salts of heavy metals, alkylating agents such as cyclophosphamide or méchlorétamine, anti-metabolites such as derivatives of uracil or cytosine, certain derivatives of the series of antibiotics such as bleomycin or daunomycin, or alkaloids such as vinblastine and vincristine.
These compounds may be used, as appropriate, either alone or in combination of two or more.
They generally have toxic side effects that limit or prohibit their use at high doses, and especially if the patient has associated defects or sequelae of mutilating surgery or radiotherapy.

[0006] All of these therapies, to varying degrees, are designed to eliminate as much or all of the cancerous tumors, but none of them exerts action on the evolutionary genius of the cancer process.

[0007] We know the importance of vitamins, water soluble and fat in nutritional physiology and biochemistry.
By cons, their use for cancer treatment, alone or in combination with other compounds, has not been described so far.

[0008] On the other hand, some amino acids are used in therapy for the treatment of various diseases.
Thus, for example it is known that 5-alanine has inhibitory properties of the peripheral vasodilatation allowing its administration for the treatment of hot flashes of menopause.
Its toxicity is low because the lethal dose DLsopar orally in mice and rats is greater than 10g/kg, and tolerance is generally satisfactory.
If the antihistamine and antiallergic activity of [beta]-alanine has been described, it does not know any cons by application of the compound, alone or in combination, to treat cancer.

[0009] It is also recognized that the [beta]-alanine acts as a neurotransmitter in the central nervous system, and could act at the cellular level in relation to the calcium signal.

[0010] We know a few compositions containing various components, including some amino acids and vitamins, for example "Unlisted Drug" vol. 28 No. 7 mentions a composition for use as a nutritional supplement, containing pyridoxine, citric acid, taurine, etc..
However, the specific use of the [beta]-alanine combined with vitamins has not been considered.

[0011] It has now been found that a composition containing [beta]-alanine in combination with various vitamins, has properties allowing its application in therapy for the treatment of cancer, whereas no comparable activity is observed when is used in isolation each of these compounds.

[0012] The present invention thus provides a new composition based on [beta]-alanine and vitamins, used to treat cancer.

[0013] The new composition in accordance with the present invention comprises at least one vitamin and [beta]-alanine represented by general formula (I):
H2N - (CH2) 2 - COOH (I)
or a salt thereof acid or base.

[0014] More particularly, the composition according to the invention comprises the [beta]-alanine of the formula (I) above and one or more vitamins selected from vitamin A, vitamin B, vitamin B2, vitamin Bs, pyridoxine, ascorbic acid, tocopherol, vitamin D2, and vitamin PP.

[0015] [beta]-alanine can be used individually or, where appropriate, in combination with one or more other amino acids, for example 5-alanine and glycine, or [beta]-alanine, and taurine, can be combined.

[0016] In addition to vitamins and the amino acid indicated above, the composition may advantageously also contain additives such as glucose, [beta]-carotene and other vitamins such as biotin and vitamin B12.

[0017] In one preferred embodiment of the invention, the composition comprises the [beta]-alanine, and more vitamins selected from those listed above, as well as glucose, and possibly taurine, and / or glycine.
It may be preferable to add to all of these constituents of P-carotene, a precursor of vitamin A.

[0018] The weight ratio of the amino acid to the total of vitamins is comprised between 50/1 and 500/1 approximately, and preferably between 100/1 and 300/1 about, and the amount of amino acid administered per day is between 50g and 200g for an etching treatment, and between 10 and 50g for maintenance therapy in adult men.
Of course, the dose is determined by the doctor depending on the patient's condition.
When glucose is used, it may represent between 10 and 30% by weight of the amino acid dose to an etching treatment.
The amount of glucose used is substantially the same in an etching treatment and maintenance therapy.

[0019] The invention also concerns a method for preparing a composition useful in therapeutic oncology.

[0020] The components constituting the composition of the present invention can be administered in combination or separately, preferably orally.
It is for example the amino acid mix, vitamins and, where appropriate, glucose and other additives in the form of a powder which can be packaged in various forms usual, for example in sachets, capsules or capsules.
It is also possible to package the amino acid separately, on the one hand, and vitamins and optional further additives, on the other hand, for example in capsules or capsules for oral administration, separately, the administration of each of these two parts can be spaced in time from minutes to several hours, but will not exceed 4-5 hours.

[0021] The composition according to the present invention can provide a therapeutic support usual therapeutic treatments, chemicals, chemotherapy or radiotherapy, in the case of cancer treatment.
The advantage of the therapy according to the present invention include the fact that taitement known, which, as noted above, are traumatic or toxic, can now be used in lower doses that avoid harmful side effects.

[0022] The experimental results showed that the composition of the invention does not destroy cancer cells, but inhibits cell division.
It has the property, such as antimitotic and radiotherapy, to act on carcinomédine, potentiating tumor cell multiplication.
It is interesting to note that these results are obtained when the components are administered simultaneously, in a mixture, but also when the vitamins on the one hand, and the amino acid, on the other hand, are administered separately at an interval of time ranging from few minutes to 4-5 hours.

[0023] For cons, no killing effect on cancer cells is observed only if administering an amino acid, or vitamins, or if their weight ratio or the doses are outside the above limits.

[0024] Toxicity studies have shown that the toxicity of the composition of the invention is no power at the doses administered cancer treatment.

[0025] Examples of compositions according to the present invention are given below without limitation.

EXAMPLE 1

[0026] A mixture of the following components listed below:

[0027] These components are carefully mixed to obtain a homogeneous composition corresponding to the dose administered 24 hours to an etching treatment.
The administration is made orally in four equal parts, every 6 hours, whether by voluntary or added to the solution of enteral nutrition.

EXAMPLE 2

[0028] The procedure of Example 1 using the components below to prepare a composition for maintenance therapy:

[0029] Thus, a quantity of composition corresponding to the dose administered within 24 hours during maintenance therapy.
The above composition is administered in four equal parts, every 6 hours.,

EXAMPLE 3

[0030] Proceeding as in Example 1 A mixture of the following components:

[0031] The mixture is homogenized and then divided among five identical bags for oral administration.
In addition, we prepare in the usual way, with 5 sachets 4g taurine.

[0032] The composition is administered at five intakes a sachet of the mixture of vitamins and [beta]-alanine above, spaced four hours, followed by an hour apart by five shots from a bag taurine.
It has an aggressive treatment that is renewed for a period which depends on the patient's clinical status.

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Notice

Claims EP0280593

1.
A composition for use in therapy, especially for the treatment of cancer, characterized in that it comprises at least one vitamin and [beta]-alanine represented by general laformule (I):
H2N - (CH2) 2 - COOH
or a salt thereof acid or base.

2.
The composition of claim 1, characterized in that it comprises one or more vitamins selected from vitamin A, vitamin B1, vitamin B2, vitamin B5, pyridoxine, ascorbic acid, tocopherol, vitamin D2, and vitamin PP.

3.
The composition of claim 1, characterized in that it further contains taurine and / or glycine.

4.
Composition according to any one of claims 1 to 3, characterized in that it further comprises at least one additive selected from glucose, [beta]-carotene, biotin and vitamin B12.

5.
Composition according to any one of claims 1 and 2, characterized in that it comprises the [beta]-alanine, taurine and / or glycine, at least one vitamin and glucose.

6.
The composition of claim 1, characterized in that the weight ratio of the total amino acid vitamins is between 50/1 and 500/1.

7.
Composition according to any one of claims 1 to 6, characterized in that it is in the form of a mixture of its components, orally administrable.

8.
Composition according to any one of claims 1 to 6, characterized in that its components are packaged separately.

9.
A process for the preparation of a therapeutic composition useful in oncological according to any one of the preceding claims, characterized in that the mixture [beta]-alanine, vitamins, and other components, if any, in the form of powder.

EXHIBIT L

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INTESTINAL ABSORPTION OF THE INTACT PEPTIDE CARNOSINE IN MAN, AND COMPARISON WITH INTESTINAL PERMEABILITY TO LACTULOSE

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(*Received 10 September 1990*)

SUMMARY

1. Healthy humans ingested the dipeptide carnosine (L-β-alanyl-L-histidine). Their plasma levels and urinary outputs of carnosine and β-alanine were monitored over the following 5 h.

2. Large amounts of intact carnosine (up to 14% of the ingested dose) were recovered in the urine over the 5 h after ingestion. However, carnosine was undetectable in the plasma unless precautions were taken to inhibit blood carnosinase activity *ex vivo* during and after blood collection.

3. The amount of carnosine recovered in urine varied substantially between subjects. It correlated negatively with carnosinase enzymic activity in the plasma. Highest carnosinase activities were observed in those subjects who regularly underwent physical training.

4. Urinary recovery of the disaccharide lactulose also varied considerably between subjects, but was substantially lower than that of carnosine. There was no significant correlation between the recoveries of carnosine and lactulose.

5. When lactulose was ingested with a hypertonic solution, the urinary recovery of lactulose was generally increased. When carnosine was ingested with a hypertonic solution, the urinary recovery of carnosine was reduced: hence the paracellular route probably is not dominant for absorption of intact carnosine.

6. Intact carnosine must have crossed the intestine to an extent much greater than hitherto recognized. Rapid post-absorptive hydrolysis is a severe obstacle to quantification of intact peptide absorption.

INTRODUCTION

Peptide transport systems in the small intestinal brush border are well documented and are thought to play a major role in absorption of the digestion products of dietary protein (e.g. Matthews, 1975, 1991). However, it is commonly assumed that peptides absorbed via these mechanisms are hydrolysed in the epithelial cytosol so that only free amino acids enter the circulation. Much evidence suggests that this

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view is incorrect (Gardner, 1984; Gardner & Wood, 1989), but it has hitherto been impossible to obtain reliable estimates of the total amounts of intact peptides that enter the circulation during assimilation of protein. This assumes special significance since (i) biologically active peptides can be produced experimentally by peptic digestion of dietary proteins (e.g. Zioudrou, Streaty & Klee, 1979), and (ii) such peptides might arise under physiological conditions in the intestinal lumen. If such peptides were absorbed, and a number of factors are now recognized to enhance intestinal 'permeability' to small and medium-sized molecules (e.g. van Hoogdalem, de Boer & Breimer, 1989), they might exert activity in peripheral tissues (e.g. Gardner, 1985). An understanding of routes and mechanisms for absorption of intact peptides is also potentially relevant for the design of strategies for oral delivery of peptide and peptide-like drugs.

In order to investigate the extent of intact peptide absorption and possible transport mechanisms, we have studied the absorption of a dipeptide, carnosine (L-β-alanyl-L-histidine; mol. wt 226), that is already believed to be absorbed to some extent in intact form (e.g. Perry, Hansen, Tischler, Bunting & Berry, 1967; Matthews, Addison & Burston, 1974). Absorption has been assessed by oral tolerance tests: plasma levels and, particularly, urinary outputs have been measured over 5 h after ingestion of a dose of carnosine by healthy humans. Since hypertonic solutions enhance permeability to various molecules including lactulose (e.g. Laker & Menzies, 1977; Wheeler, Menzies & Creamer, 1978), supposedly absorbed via the paracellular route, we have investigated the effect of hypertonic solutions on the urinary recovery of carnosine. Most of our test meals included also the monosaccharide rhamnose (mol. wt 164) and the disaccharide lactulose (4-O-β-D-galactopyranosyl-D-fructose; mol. wt 342) since these are now widely used as probes of intestinal 'permeability' (e.g. Hamilton, 1986).

After our early experiments suggested that hydrolysis of carnosine in blood might be a determinant of urinary recovery, we measured hydrolytic activity against carnosine ('carnosinase') in plasma. Since early data suggested that the exercise status of the subjects might be relevant, we classified the subjects as 'exercisers' or 'non-exercisers'.

Preliminary communications have described part of this work (Gardner & Wood, 1987, 1989).

METHODS

Subjects

Nine healthy subjects, male and female, aged between 23 and 50 years participated. They gave informed consent, and the study was approved by a University of Bradford ethics committee. Eight subjects had no history of gastrointestinal disease; one had undergone 11 years previously a truncal vagotomy and pyloroplasty for gastric stress ulceration following a hypotensive crisis. Five subjects ('exercisers') regularly undertook formal exercise; the other four ('non-exercisers') did not.

Experimental procedure

Subjects fasted overnight from 23.00 h before an experiment. At the start of the experiment, between 08.30 and 09.30 h the next day, subjects emptied their bladders and ingested a test meal which was designated (a) 'blank', (b) 'isotonic' (nominally) or (c) 'hypertonic'. All subjects

consumed at least one of each test meal, and at least 1 week elapsed before anyone underwent another experiment. The 'blank' meal comprised 92·5 ml hot water, 7·5 ml Duphulac syrup (Duphar Laboratories Ltd, Basingstoke, Hants, UK) and 1 g rhamnose (Sigma Chemical Co.). The Duphulac contained 5 g lactulose and traces of galactose and lactose in aqueous solution. The 'isotonic' test meal contained the same ingredients with the addition of 4 g carnosine (Sigma Chemical Co.). (In some experiments on one subject, smaller doses of carnosine were ingested.) The osmotic pressure of this solution was 344 mosmol/kg measured cryoscopically. The 'hypertonic' test meal was the same as the 'isotonic' one, but with the addition of 35 g lactose and 35 g sucrose (both from Sigma Chemical Co.); it had an osmotic pressure of approximately 2300 mosmol/kg. (In an additional experiment on one subject, an approximately isotonic test meal containing 2 g β-alanine plus 2 g histidine instead of carnosine was taken. In another experiment, the test meal contained 0·4 g of glycyl-sarcosine supplied by Sigma Chemical Co. in place of carnosine.)

Subjects were allowed free intake of water or orange juice (recommended to be 200–500 ml per hour) during the experiment. Urine was collected either every hour for five hours or over the 5 h period into bottles containing 10% w/v thiomersal (0·2 or 1·0 ml, respectively).

In some experiments, a cannula was inserted in a superficial vein in the forearm before the start of experiment, and blood samples (10 ml) were taken at the following times after the test meal: 0, 30, 60, 90, 120, 150, 180 and 360 min. Blood was transferred to lithium heparin tubes, and plasma was separated rapidly. In one experiment, the syringes, anticoagulant tubes, and the centrifuge were pre-chilled to 4 °C. All plasma samples were stored at −20 °C pending assay.

Analytical methods

Carnosine, β-alanine and glycyl-sarcosine. These were determined on an ion-exchange amino acid analyser with ninhydrin detection ('Alpha-Plus', L.K.B. Instruments Ltd). Plasma or urine (1 ml) was deproteinized with sulphosalicylic acid (0·1 ml; 30 mg/ml) containing norleucine (100 nmol/ml) as internal standard. Eighty microlitres of the supernatant were loaded onto the analyser, and a standard 'physiological fluid' programme with lithium citrate buffers was used. Carnosine emerged as a single peak at approximately 116 min, and analyses of 'blank' urine specimens indicated that it did not co-elute with any other ninhydrin-positive constituent in the urine. β-Alanine eluted at approximately 78 min, and glycyl-sarcosine at 60 min. Peak areas were integrated and were related to both external and internal standards.

Lactulose and rhamnose. Urine (50 μl) with inositol as internal standard was analysed by gas–liquid chromatography as follows. Oxime derivatives of the sugars were formed by incubation with 100 μl 2·5% ethoxylamine in pyridine at 60 °C for 30 min followed by trimethylsylation with 20 μl hexamethyldisilazane and 20 μl trimethylchlorosilane at 60 °C for 30 min. Samples were dried on a sand bath at 60 °C under a stream of N_2 and taken up in 1 ml heptane. One microlitre was injected onto an SE-54 capillary column (30 m × 0·25 mm i.d. splitless mode) at an injection temperature of 250 °C and flame-ionization detector temperature of 350 °C. The initial separation temperature, 80 °C, was maintained for 2 min followed by a stepwise rise to 290 °C at 16 °C/min at a column head pressure of 0·8 bar. The recovery (±S.D.) of lactulose added to urine (10 samples) was 97·2±6·2%; the between-batch coefficient of variation was 4·0%.

Plasma carnosinase activity. This was assayed by the method of Bando, Shimotsuji, Toyoshima, Hayashi & Miyai (1984) as modified by Bando, Ichihara, Shimotsuji, Toyoshima, Koda, Hayashi & Miyai (1986) in which the appearance of free histidine is measured fluorimetrically following incubation of the plasma with carnosine at 37 °C.

RESULTS

Urinary output of carnosine

Subjects not ingesting a carnosine test meal excreted 24·0±2·36 μmol carnosine in 5 h ($n = 9$). No adverse effects were caused by carnosine ingestion although some subjects experienced mild and transient digital paraesthesia within the first hour, a similar effect having been reported by Asatoor, Bandoh, Lant, Milne & Navab (1970). Figure 1 shows the urinary content of carnosine over the first 5 h after

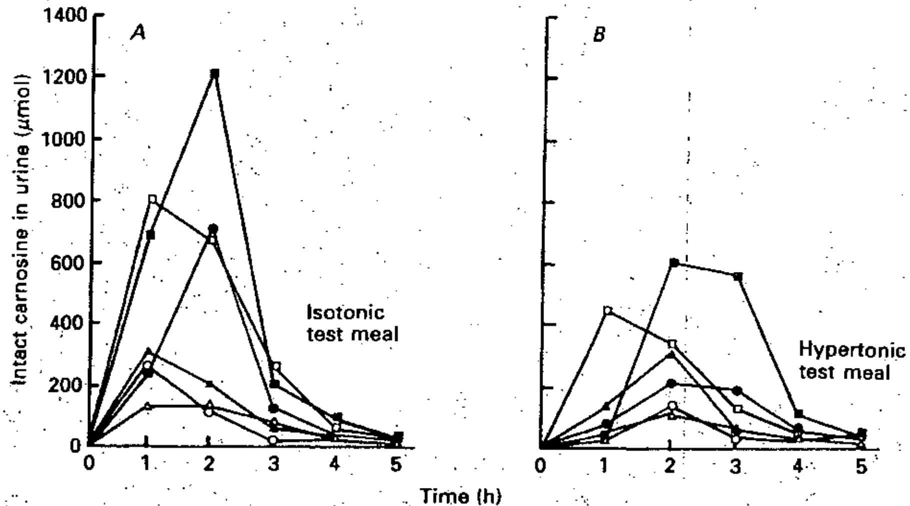

Fig. 1. Hourly urinary output of intact carnosine following ingestion of 4 g carnosine with an isotonic (*A*) or a hypertonic (*B*) test meal. Each symbol represents a different subject.

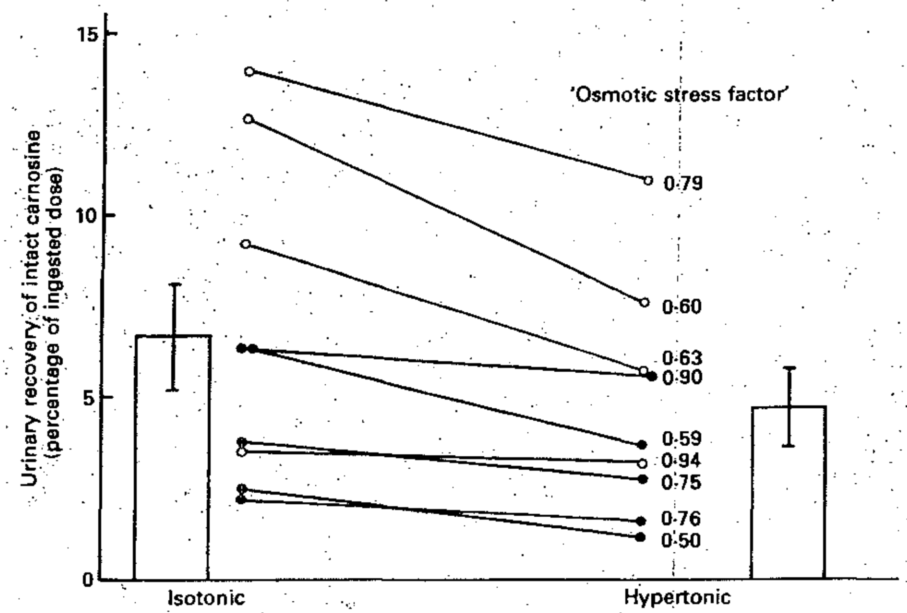

Fig. 2. Urinary recoveries of intact carnosine for 5 h after ingestion of 4 g carnosine with either an isotonic or a hypertonic test meal. Each pair of points represents a single subject. The filled symbols (●) represent 'exercisers' and the open symbols (○) represent 'non-exercisers'. The bars show the mean value ± S.E.M. for the nine subjects.

ingestion of 4 g of carnosine by six subjects. It was maximal at 1–2 h and returned to basal levels by 5 h after both the isotonic (Fig. 1*A*) and hypertonic (Fig. 1*B*) test meals. The area under the curve varied substantially between subjects. The recovery

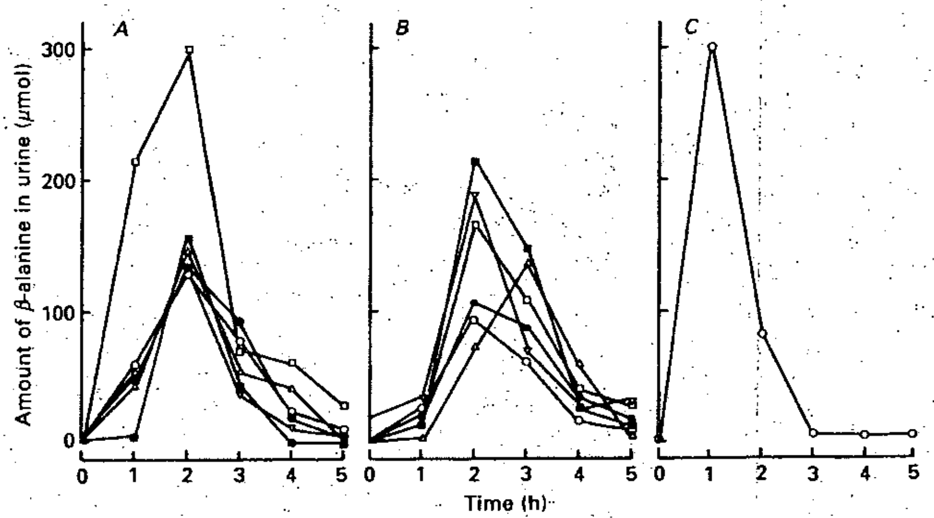

Fig. 3. Urinary output of β-alanine for 5 h after ingestion of 4 g carnosine in an 'isotonic' test meal (six subjects) (*A*), 4 g carnosine in a 'hypertonic' test meal (six subjects) (*B*) and 2 g L-histidine plus 2 g L-β-alanine (one subject) (*C*). Each symbol represents a different subject. In some cases overlapping symbols have been omitted for clarity.

TABLE 1. Reproducibility of urinary carnosine recovery by four subjects undergoing replicate experiments

	Isotonic meal (%)	Hypertonic meal (%)
Subject 1	10·3; 9·4; 7·1; 6·7	5·5; 5·2; 6·5
Subject 2	3·6; 3·8	2·7; 2·8
Subject 3	6·4; 6·3	3·1; 4·4
Subject 4	1·5; 1·8	

(percentage of ingested dose) varied between subjects from 1·2 to 14·0% (197 to 2480 μmol; see Fig. 2). Figure 2 shows also that the recovery of intact carnosine was less for each of the nine subjects when the test meal was made hypertonic (approx. 2300 mosmol/kg). The effect of tonicity has been expressed as an 'osmotic stress factor' (urinary recovery after hypertonic meal divided by recovery after isotonic meal) and, for carnosine, this ranged from 0·5 to 0·94 (Fig. 2). The correlation between the recoveries under isotonic and hypertonic conditions was highly significant ($r = 0{\cdot}958$; $P < 0{\cdot}001$). The amount of β-alanine (a product of carnosine hydrolysis) appearing in the urine over 5 h corresponded to approximately 2% of the ingested carnosine, and this was much higher than the basal excretion of β-alanine (Fig. 3*A* and *B*).

Four subjects performed replicate experiments. The limited data from these showed that the urinary recovery of carnosine was acceptably reproducible for each subject (Table 1). One subject also consumed test meals containing 1, 2 or 4 g of carnosine. Urinary recoveries were 1·3, 2·3 or 3·4% respectively of the consumed doses. In a single experiment to investigate Block, Hubbard & Steele's (1965)

suggestion that the urinary carnosine might have arisen from resynthesis of carnosine after gastrointestinal hydrolysis, one subject ingested a test meal containing histidine (2 g) and β-alanine (2 g). Only traces (approximately basal amounts) of carnosine were detected in the urine; substantial amounts of β-alanine

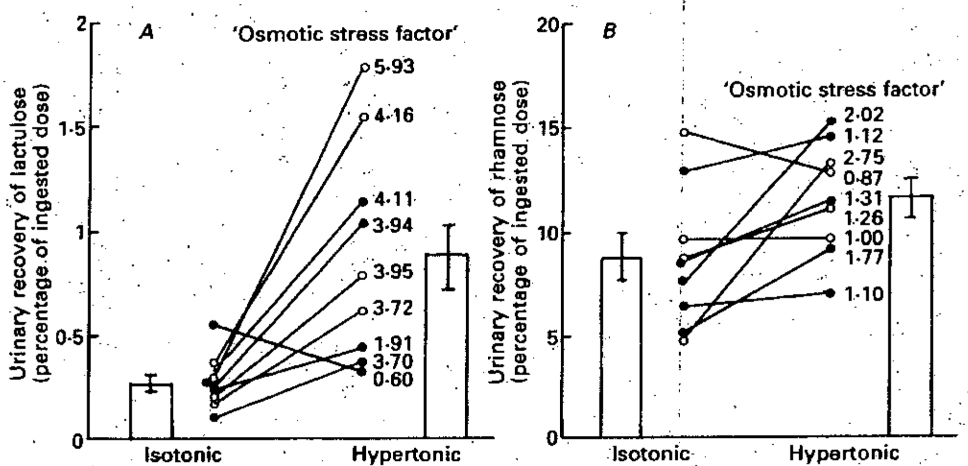

Fig. 4. Urinary recoveries of lactulose (A) and rhamnose (B) for 5 h after ingestion of an isotonic or a hypertonic test meal. Each pair of points represents a single subject. The filled symbols (●) represent 'exercisers' and the open symbols (○) represent 'non-exercisers'. The bars show the mean value ± S.E.M. for the nine subjects.

appeared in the urine (corresponding to approximately 2% of the ingested dose over 5 h; see Fig. 3C).

Urinary output of lactulose and rhamnose

Figure 4 shows the urinary recoveries of lactulose and rhamnose for each subject. Lactulose recoveries ranged from 0·1 to 0·55% of the ingested dose when the test meal was isotonic and from 0·33 to 1·78% when it was hypertonic. In eight of the nine subjects the hypertonic meal caused an increase in lactulose recovery, their 'osmotic stress factors' ranging from 1·91 to 5·93 (Fig. 4A). One subject ingested six isotonic and six hypertonic test meals over a 12 week period, and his lactulose recoveries were always higher after the hypertonic test meal than after the isotonic one ($1{\cdot}14 \pm 0{\cdot}200$ *vs.* $0{\cdot}292 \pm 0{\cdot}0306$% respectively; $P < 0{\cdot}01$). Figure 4B shows that the rhamnose recoveries for all subjects ranged from 4·84 to 15·25%, and the effect of the hypertonic test meal was small and less consistent though with a trend towards an increase. A significant increase was, however, seen for the subject undertaking the replicate experiments: $11{\cdot}35 \pm 0{\cdot}559$% ($n = 6$) after a hypertonic meal *vs.* $8{\cdot}74 \pm 0{\cdot}552$% ($n = 6$) after an isotonic meal ($P < 0{\cdot}01$).

Correlations between recoveries of carnosine and lactulose or rhamnose

No significant correlations were observed between the urinary recovery of carnosine and that of either lactulose or rhamnose under either isotonic or hypertonic conditions.

Urinary recovery of glycyl-sarcosine

In a single experiment a subject ingested 0·4 g of glycyl-sarcosine (glycyl-*N*-methyl-glycine) in 100 ml of water. Hourly urine collections showed that 10·5 % was recovered in 6 h, with the maximum amount (6 % of the ingested dose) being

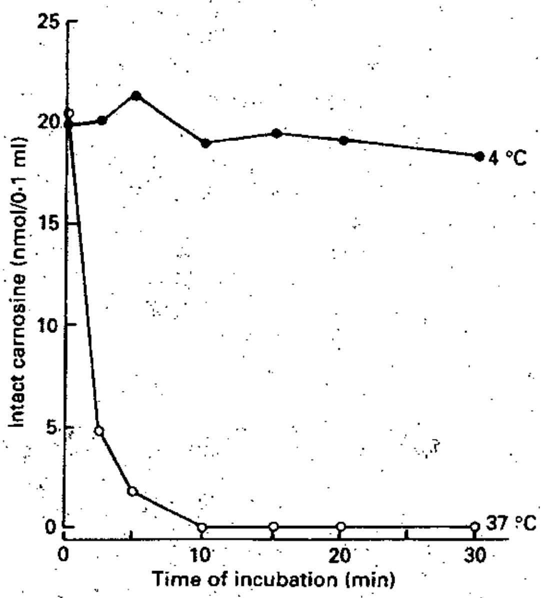

Fig. 5. Time course of disappearance of carnosine after addition to plasma *in vitro*. Samples were incubated either at 37 °C (○) or on ice (●).

excreted in the first hour. No sarcosine was detected on the amino acid chromatogram. Incubation of glycyl-sarcosine with blood or plasma for 40 min at 37 °C showed negligible hydrolysis.

Plasma levels of carnosine and carnosinase activity

In initial experiments, no carnosine was detected in plasma (limit of detection approx. 5 nmol/ml) after carnosine ingestion even though large amounts were found in corresponding urine specimens. The reason for this is apparent from Fig. 5 which shows the recovery of carnosine added *in vitro* to plasma and incubated at either 4 or 37 °C. This suggests that enzymic activity in plasma hydrolyses the added carnosine with a half-life of the order of 1 min. A further experiment on a subject who had ingested carnosine (4 g) showed that small amounts of carnosine (81·1, 44·4, 4·6 nmol/ml plasma) could be detected in the blood (at 0·5, 1·0, 1·5 h respectively) if the collection syringe, specimen bottle and centrifuge were chilled. Further attempts to measure plasma levels of carnosine were abandoned owing to the difficulties of halting peptide hydrolysis *ex vivo* during blood collection.

Carnosinase activity in plasma from each subject was measured, and this showed wide variability. Figure 6 shows that there is a highly significant negative correlation

between the plasma carnosinase activity and the urinary recovery of intact carnosine ($r = -0.815$; $P = 0.004$). These data are for the isotonic test meal, but a similar correlation was observed also for the hypertonic test meal ($r = -0.787$; $P = 0.006$).

DISCUSSION

Although it has previously been shown that intact carnosine appears in urine after ingestion of rabbit or chicken meat (e.g. Block *et al.* 1965; Perry *et al.* 1967), to our

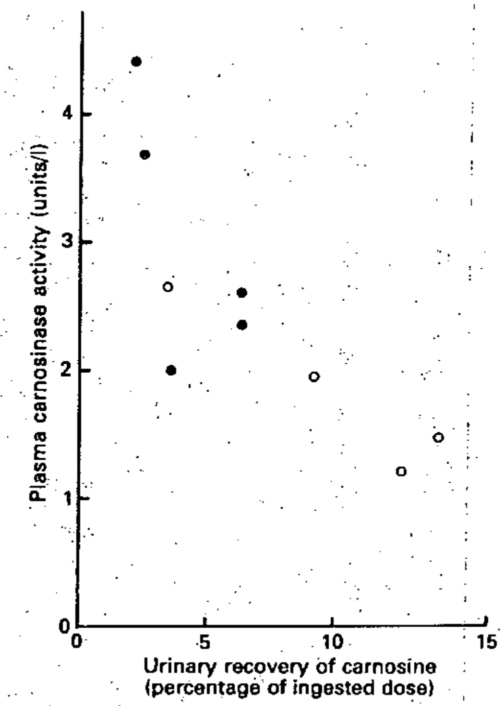

Fig. 6. Relationship between each subject's carnosinase activity in plasma and the urinary recovery of intact carnosine during the 5 h after ingestion of 4 g of carnosine with an isotonic test meal. The correlation is significant ($r = -0.815$; $P = 0.004$). A similar relationship was seen for the recovery of carnosine after the hypertonic test meal. Each point represents a different subject. Filled symbols (●) represent 'exercisers' and open symbols (○) represent 'non-exercisers'.

knowledge this is the first time that this process has been quantified. Mechanisms have been found for the active transport of carnosine in the brush border of rat and hamster small intestine, competition studies suggesting that the transporter is shared by some other peptides and by amino-β-lactam antibiotics (e.g. Matthews *et al.* 1974; Tsuji, 1987). The brush-border transport of carnosine is said to be regulated by dietary levels of amino acids, peptides and/or proteins (Ferraris, Diamond & Kwan, 1988). A putative proton-linked peptide carrier has also recently been partially characterized in basolateral membranes, and carnosine inhibited the transport of glycyl-L-proline by it (Dyer, Beechey, Gorvel, Smith, Wootton &

Shirazi-Beechey, 1990). However, carnosinase activity has been identified in the small intestine of several species including humans (Parshin, 1946; Sadikali, Darwish & Watson, 1975) though Parshin (1946) found less activity in human mucosa than in other species, and Hanson & Smith (1949) reported that it was absent from pig intestine though present in pig kidney.

Our results show that up to 14% of ingested carnosine may be excreted in intact form and, thus, must have been absorbed across the gastrointestinal tract in intact form (Fig. 2). The experiment entailing ingestion of histidine plus β-alanine eliminates the possibility that the urinary carnosine had arisen from resynthesis after hydrolysis of the ingested peptide. In the experiment performed on glycyl-sarcosine, a hydrolysis-resistant dipeptide (Addison, Burston & Matthews, 1972), 10·5% of the intact peptide was recovered in urine. In view of the remarkably short half-life of carnosine in plasma (Fig. 5), it is very likely that the amounts crossing the gastrointestinal tract in intact form greatly exceed the amounts collected in the urine. (Indeed, it is possible that a majority of, or even all, the carnosine had been absorbed in intact form.) The high negative correlation between the urinary recovery of the peptide and the carnosinase activity in each subject's plasma (Fig. 6) strongly suggests that this enzymic activity has a dominating effect on the urinary recovery and that the wide variation in urinary recoveries between subjects (Fig. 2) is predominantly influenced by the subjects' carnosinase activities. The lack of correlation between carnosine recovery and recovery of lactulose or rhamnose supports the view that extra-intestinal factors primarily determine the urinary recovery of carnosine.

The presence of such effective carnosinase activity in plasma explains why Asatoor *et al.* (1970) and Sadikali *et al.* (1975) failed to detect carnosine in plasma after carnosine ingestion by humans. It also emphasizes the danger in using plasma measurements as an index of intestinal absorption of intact peptides. Further, systemic hydrolysis may therefore be as serious an obstacle to the efficacy of peptide-like drugs as is intraluminal hydrolysis or poor intestinal absorption of such drugs.

It is impossible to estimate the role of intestinal carnosinase in the hydrolysis of carnosine but it may well be minimal compared to hydrolysis in blood, liver, kidney and other tissues. Sadikali *et al.* (1975) concluded that intestinal hydrolysis of carnosine was the rate-limiting step in carnosine absorption. This conclusion was based mainly on the observation that a patient with a deficiency of intestinal carnosinase produced a relatively low serum histidine tolerance curve after ingestion of carnosine. However, an equally plausible explanation would be that the subject, who had sub-total villous atrophy associated with untreated coeliac disease, had a deficiency of the relevant peptide carrier(s). It thus appears that intestinal hydrolysis of carnosine may be irrelevant to the absorption of carnosine, and certainly it does not appear to be a rate-limiting step.

These results pose a question as to the fate of the absorbed carnosine before it is excreted. Figure 1 suggests that intestinal absorption is fairly rapid, as would be expected from such a test meal in fasted subjects. However, carnosine is still being excreted by the fourth hour after ingestion. In the light of the very short half-life in blood (Fig. 5), it seems that absorbed carnosine may be very rapidly cleared from the plasma and sequestered in some compartment before it is excreted by the kidneys.

The identity of such a compartment is unknown, but the evidence of Hama, Tamaki, Miyamoto, Kita & Tsunemori (1976), who fed high doses of carnosine to rats, would favour accumulation in liver rather than muscle even though skeletal muscle is the major repository of endogenous carnosine.

The hypertonic test meal caused a substantial increase (up to 6-fold) in the lactulose recovery in all subjects except one, and there was marked variation in the effect of hypertonicity (see Fig. 4*A*). This is in agreement with the observations of Menzies (1974, 1984), Wheeler *et al.* (1978) and Maxton, Bjarnason, Reynolds, Catt, Peters & Menzies (1986), and is probably due to enhancement of paracellular permeability via the 'tight' junctions. Temporary cellular shrinkage might be responsible. However, it is noteworthy that hypertonic solutions had the converse effect on recovery of carnosine (Fig. 2): in all nine subjects a modest reduction was observed. Hence, it is considered unlikely that the paracellular route is an important one for carnosine absorption; however, this could be a reflection of the relative efficiency of the brush-border transport mechanism for carnosine. It may be significant that, when Fuessl, Domin & Bloom (1987) demonstrated biological activity of an octapeptide mini-analogue of somatostatin (Sandoz SMS-201-995) after oral administration to humans, they administered it with a strongly hypertonic glucose solution (2100 mosmol/kg nominally). Hence, hypertonic solutions may enhance intestinal absorption of intact peptides, but this may be relevant only when the specificity of brush-border carriers and/or the size of the peptides denies the use of transcellular carrier-mediated mechanisms.

Rhamnose, now used routinely by some workers as a probe for investigation of intestinal permeability in humans, is presumed to be absorbed predominantly via the transcellular route (Menzies, 1984). Figure 4*B* shows that hypertonic solutions did not have a consistent effect on urinary recovery of this monosaccharide though a marked (nearly 3-fold) increase was produced in some subjects and a significant 30% increase ($P < 0.01$) was observed in the one subject undertaking replicate experiments. It is useful to note that there was no correlation between the rhamnose and lactulose recoveries ($r = -0.2346$ and 0.1710 for isotonic and hypertonic conditions respectively, with sixteen degrees of freedom), which suggests that the variability in these was not predominantly caused by factors common to both rhamnose and lactulose recoveries (e.g. gastric emptying, transit time, extracellular volume, renal function etc.; see Menzies, 1984). This is further supported by the fact that the 'between-subject' coefficient of variation for the (lactulose/rhamnose) recovery ratio (71%) is greater than that for either lactulose recovery (38%) or rhamnose recovery (48%) alone, even though clinical experience has consistently shown the lactulose/rhamnose ratio to have better power than lactulose or rhamnose recovery alone to discriminate between diseased and healthy subjects (e.g. Figs 45.6 and 45.7 of Menzies, 1984).

Although this work was primarily intended to quantify and elucidate mechanisms of peptide absorption, two incidental findings of interest are that variability in plasma carnosinase activity may be related to the subject's exercise status (Fig. 6) and that variability in permeability to lactulose may be related to lean body mass or to protein turnover (M. L. G. Gardner & D. Wood, unpublished observations). The former would accord with the views inherent in the work of Bando *et al.* (1984)

and Duane & Peters (1988) that carnosinase is involved in muscle turnover, and this could explain why excretion of absorbed carnosine tended to be lower in our 'exercisers' than in our 'non-exercisers' (Fig. 2). Both these issues merit fuller investigation.

We are indebted to our friends and colleagues who acted as experimental subjects and to Mr T. K. Hepworth for technical assistance. M. L. G. G. records his gratitude to the late Professor D. M. Matthews for many valuable discussions on peptide absorption.

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EXHIBIT M

US005869068A

United States Patent [19]

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[54] **COMPOSITIONS AND METHODS FOR TREATING WRINKLES AND/OR FINE LINES OF THE SKIN**

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[52] **U.S. Cl.** **424/401**; 514/844; 514/846; 514/944

[58] **Field of Search** 424/401; 514/844, 514/846, 944

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[57] **ABSTRACT**

Compositions which contain an agonist substance of one or a number of receptors associated with a chlorine channel are useful for slackening and/or relaxing cutaneous tissue, and in particular for the purpose of treating wrinkles and fine lines of the skin. Such compositions can be administered topically or by injection. Preferred agonists include glycine, serine, taurine, β-alanine, N-(benzyloxycarbonyl)glycine (Z-glycine), gamma-aminobutyric acid (GABA), isoguvacine, isonipecotic acid, 4,5,6,7-tetrahydroisoxazolo [5,4-c]pyrid-3(2H)-one, benzodiazepines, steroids, and barbiturates. The composition can additionally contain a retinoid and/or a hydroxy acid.

34 Claims, No Drawings

COMPOSITIONS AND METHODS FOR TREATING WRINKLES AND/OR FINE LINES OF THE SKIN

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to the use of substances which are agonists of a receptor associated with a chlorine channel in a cosmetic and/or dermatological composition, in particular for the purpose of treating wrinkles and fine lines of the skin, and to cosmetic and/or dermatological compositions which contain such a substance.

Discussion of the Background

Women, and indeed even men, are currently inclined to wish to appear young for as long as possible and consequently are looking to soften the signs of ageing of the skin, which are reflected in particular by wrinkles and fine lines. In this respect, advertising and fashion present products intended to retain a radiant and wrinkle-free skin, these being the signs of young skin, for as long as possible, all the more so since physical appearance has an effect on mental attitude and/or on morale. It is consequently important to feel physically and spiritually young.

Until now, wrinkles and fine lines have been treated using cosmetic products containing active agents which act on the skin, for example by moisturizing it or by improving its cell renewal or alternatively by promoting the synthesis of collagen of which the cutaneous tissue is composed. However, to date, it is not known to act on wrinkles by involving the muscle components present in the skin.

It is known that the platysma muscles of the face are under the control of the motor nerve afferent activity of the facial nerve and that, moreover, the interlobular septa of the hypoderm contain within them fibers which constitute a striated muscle tissue (panniculus carnosus). Moreover, it is also known that a subpopulation of fibroblasts of the dermis, known as myofibroblasts, has characteristics in common with the muscle tissue.

The Applicants have observed, in certain pathological and therapeutic situations, the role played, as regards the wrinkles of the face, by the nerves controlling all this muscle tissue. Thus, in attacks on the facial nerve, in which transmission of the nerve impulse is interrupted and/or weakened, a paralysis of the muscles of the face is witnessed in the area of innervation. This facial paralysis is reflected, among other clinical indications, by an alleviation in, indeed disappearance of, the wrinkles.

On the other hand, in muscle hypercontraction conditions of the face, the Applicants have observed an accentuation in the wrinkles of the face. Moreover, an accentuation in the wrinkles of the face has also been observed in muscle hypertonia conditions of Parkinson's disease and side-effects induced by neuroleptics.

Moreover, it has been shown that botulinus toxin, originally used for treating spasms, could have an effect on muscle spasticity conditions (see A. Blitzer et al., *Arch. Otolaryngol. Head Neck Surg.*, vol 119, pages 1018 to 1022 (1993)) and on the wrinkles of the glabella, which are intersuperciliary wrinkles (see J. D. Carruters et al., *J. Dermatol. Sura. Oncol.*, vol. 18, pages 17 to 21 (1992)). It is consequently possible, by pharmacological action, to have an effect on the nerve component of wrinkles. Botulinus toxin acts directly at the level of the neuro-muscular junction by blocking the action of acetylcholine on muscular tenseness.

The junction between a nerve and a muscle constitutes the myoneural endplate, before which is found the afferent nerve route known as the motor neuron. Moreover, the cell membranes of each nerve fiber contain many ionic channels, and in particular chlorine channels, capable of allowing the corresponding element to pass through in the ionic form, and, in the case of chlorine channels, in the chloride form. Neuronal receptors are associated with these channels. The neuronal receptors associated with the chlorine channels are in particular receptors for glycine (glycine-strychnine sensitive receptors) and receptors for GABA ($GABA_A$ receptors).

Moreover, it is known that, in the central nervous system, it is possible to decrease the excitability of the neuron by various pharmacological agents which have an effect on the glycine-strychnine sensitive receptors or on the $GABA_A$ receptors of the central nervous system (see W. Sieghart, *Trends in Pharmacological Science*, vol. 131, pages 446 to 450 (December 1992)). Activation of these receptors opens the chlorine channels and leads to the entry of chloride ions, which results in an increase in the chloride ions in the cells of the nerve fiber and thus to hyperpolarization of the neurons, which consequently become less excitable.

On the other hand, in the neuromuscular junction, a decrease in excitability of the motor neuron leads to a lessened stimulation of the muscle fiber, thus causing it to slacken.

However, to date no completely suitable compositions or methods are available for treating wrinkles and/or fine lines of the skin. Thus, there remains a need for methods and compositions effective for treating wrinkles and/or fine lines of the skin.

SUMMARY OF THE INVENTION

Accordingly, it is one object of the present invention to provide novel compositions for treating wrinkles and/or fine lines of the skin.

It is another object of the present invention to provide novel methods for treating wrinkles and/or fine lines of the skin.

These and other objects, which will become apparent during the following detailed description, have been achieved by the inventors' discovery that contractile muscle fibers, which are under the direct control of the neuromotor impulse, play an essential role in the pathogenesis of wrinkles and that suppression of the neuromotor impulse alleviates not only wrinkles but also fine lines and also has a "smoothing" effect on the cutaneous microrelief. It has also been found that cutaneous tissues contain receptors associated with chlorine channels, something which, until now, had not been envisaged. It has thus been found that it is possible to act on these channels in order to slacken or relax these tissues and thus to lessen wrinkles and fine lines.

Until now, a connection between the chlorine channels of nerve fibers of the peripheral cutaneous nervous system and wrinkles had never been established, nor had it been found that it was possible to treat wrinkles by acting on chlorine channels by activation of the receptors which are found in or in the neighborhood of these channels. Substances which can activate the receptors of chlorine channels and thus lead to the entry of chloride into cells are known as agonist substances.

Consequently, the present invention provides topical cosmetic or dermatological compositions, which contain at least one agonist substance of at least one receptor associated with at least one chlorine channel present in cutaneous tissue

except glycine and gamma-aminobutyric acid for relaxing and/or slackening cutaneous tissue.

In another aspect, the present invention provides injectable cosmetic or dermatological compositions, for the purpose of lessening wrinkles and/or fine lines, which contain at least one agonist substance of at least one receptor associated with at least one chlorine channel present in cutaneous tissue for relaxing and/or slackening cutaneous tissue. In this context, the term "injectable" means suitable for injection into tissue, and in particular in wrinkles.

The present invention further provides injectable or topical cosmetic or dermatological compositions, which contain at least one agonist substance of at least one receptor associated with at least one chlorine channel of at least one cutaneous afferent nerve pathway for relaxing and/or slackening cutaneous tissue.

The present invention additionally provides topical cosmetic or dermatological compositions for the purpose of lessening wrinkles and/or fine lines which contain at least one agonist substance of at least one receptor associated with at least one chlorine channel of at least one cutaneous afferent nerve pathway, except glycine and gamma-butyric acid, for relaxing and/or slackening cutaneous tissue.

The compositions containing the agonist according to the present invention can be applied topically or by subcutaneous and/or intradermal and/or "intrawrinkle" injection.

Another aspect of the present invention is a method for the cosmetic treatment of wrinkles and/or fine lines in humans by injecting a composition containing at least one agonist substance of at least one receptor associated with at least one chlorine channel present in cutaneous tissue.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

A number of receptors associated with the chlorine channel exist. They concern in particular glycine strychnine sensitive receptors and $GABA_A$ receptors, the latter themselves containing a number of subunits comprising the GABA site, the benzodiazepine site, a type of steroid site and the barbiturates site. All the substances which act as agonists of these receptors or sites can be used for slackening or relaxing cutaneous tissues in accordance with the present invention.

For a substance to be recognized as an agonist of a receptor of the chlorine channel, it must exhibit the following two characteristics:

(i) to be able to be bound selectively to at least one of the various receptors associated with the chlorine channel; and

(ii) to show a relaxation effect on a contracted muscle tissue.

The first characteristic, which consists of the possibility of being bound to a receptor associated with a chlorine channel, does not make it possible to distinguish an agonist activity from an antagonist activity but it does make it possible to define a potential affinity for the receptor.

The second characteristic makes it possible to select the agonists. The agonist activity of the substance under study can be demonstrated by the relaxation effect which it produces on a muscle tissue which has been contracted beforehand by a chlorine channel antagonist substance. Substances known as chlorine channel antagonists can be chosen as such and in particular include the following substances: bicuculline, strychnine, tert-butylbicyclophosphorothionate and picrotoxin.

Mention may be made, as agonist substances, which can be used in the present invention for activating glycine-strychnine sensitive receptors, of glycine, serine, taurine, β-alanine, and N-(benzyloxycarbonyl)glycine or (Z-glycine).

Mention may be made, as agonist substances, which can be used in the invention for activating $GABA_A$ receptors, of gamma-aminobutyric acid (GABA), isoguvacine, isonipecotic acid, 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyrid-3(2H)-one (THIP), benzodiazepines such as nitrazepam (1,3-dihydro-7-nitro-5-phenyl-2H-1,4-benzodiazepin-2-one), diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one), flunitrazepam (5-(2-fluorophenyl)-1,3-dihydro-1-methyl-7-nitro-2H-1,4-benzodiazepin-2-one) or oxazepam (7-chloro-1,3-dihydro-3-hydroxy-5-phenyl-2H-1,4-benzodiazepin-2-one), certain steroids such as alfaxalone (3-hydroxypregnane-11,20-dione) or barbiturates such as barbital (5,5-diethylbarbituric acid), pentobarbital (5-ethyl-5-(1-methylbutyl)barbituric acid) or phenobarbital (5-ethyl-5-phenylbarbituric acid), and their salts.

It is certainly known to use GABA and glycine in combination with other active agents for combating ageing of the skin but, until now, their action in relaxing and slackening cutaneous tissues for the purpose of treating wrinkles was not known. The generally known actions are inhibition of elastase, the effect on collagen, and cell renewal.

Indeed, it is known in the state of the art to use amino acids as moisturizing agents for the purpose of improving the condition of the skin. In particular, combinations of amino acids such as glycine, taurine or β-alanine in the form of peptide mixtures have been used in cosmetic compositions intended for treating the ageing of the skin. Thus, FR-A-2,546,164 discloses the elastase-inhibiting properties of lipopeptides which prevent deterioration of elastin fibers in the skin, which makes them antiwrinkle active agents. Moreover, U.S. Pat. No. 5,198,465 discloses that amino acids prevent deficiencies in the synthesis of collagen, which consequently makes it possible to prevent ageing of the skin.

In addition, JP-A-05043448 discloses that the combination of GABA and of diisopropylamine facilitates renewal of the skin and thus prevents cutaneous ageing.

In the compositions according to the present invention, the agonist of a receptor associated with the chlorine channel is preferably used in an amount ranging from 0.00001 to 20% by weight, based on the total weight of the composition, and in particular in an amount ranging from 0.01 to 10% by weight, based on the total weight of the composition.

The compositions according to the present invention can be provided in all the pharmaceutical dosage forms normally used for a topical or injectable application.

The amounts of the various constituents of the compositions according to the present invention are those conventionally used in the fields under consideration and are appropriate to their pharmaceutical dosage form.

For topical application, the compositions of the present invention comprise a medium compatible with skin. These compositions can be provided in particular in the form of aqueous, alcoholic or aqueous/alcoholic solutions, of gels, of water-in-oil or oil-in-water emulsions having the appearance of a cream or of a gel, of microemulsions or of aerosols or alternatively in the form of vesicular dispersions containing ionic and/or nonionic lipids. These pharmaceutical dosage forms are prepared according to the conventional methods in the fields under consideration.

The present compositions for topical application can constitute in particular a cosmetic or dermatological

protection, treatment or care composition for the face, for the neck, for the hands or for the body (for example day creams, night creams, sun creams or oils or body milks), a make-up composition (for example foundation cream), or an artificial tanning composition.

When the composition of the present invention is an emulsion, the proportion of fatty substance which it contains can range from 5% to 80% by weight, and preferably from 5% to 50% by weight, based on the total weight of the composition. The fatty substances and the emulsifiers used in the composition in the emulsion form are chosen from those conventionally used in the cosmetic or dermatological field.

Mention may be made, as fatty substances which can be used in the present invention, of mineral oils (paraffin), vegetable oils (karite butter liquid fraction) and their hydrogenated derivatives, animal oils, synthetic oils (perhydrosqualene), silicone oils (dimethylpolysiloxane), and fluorinated oils. Mention may also be made, as other fatty substances, of fatty alcohols (cetyl alcohol or stearyl alcohol), fatty acids (stearic acid), and waxes.

The emulsifiers can be present in the present compositions in a proportion ranging from 0.3% to 30% by weight, and preferably from 0.5 to 30% by weight, based on the total weight of the composition.

In a conventional way, the cosmetic or dermatological compositions of the present invention can also contain adjuvants which are typical in the corresponding fields, such as hydrophilic or lipophilic gelling agents, preservatives, antioxidants, solvents, fragrances, fillers, screening agents, and colorants. Moreover, these compositions can contain hydrophilic or lipophilic active agents. The amounts of these various adjuvants or active agents are those conventionally used in the cosmetics or dermatological field and, for example, from 0.01% to 20% of the total weight of the composition. These adjuvants or these active agents, depending on their nature, can be introduced into the fatty phase, into the aqueous phase and/or into lipid vesicles.

Mention may especially be made, among the active agents which the compositions of the invention can contain, of active agents having an effect on the treatment of wrinkles or of fine lines and in particular of keratolytic active agents. The term "keratolytic active agent" is understood to mean an active agent having desquamative, exfoliative or scrubbing properties or an active agent capable of softening the corneal layer.

Mention may in particular be made, among these active agents having an effect on the treatment of wrinkles or fine lines which the compositions of the invention can contain, of hydroxy acids and retinoids.

The hydroxy acids can be, for example, α-hydroxy acids or β-hydroxy acids, which can be linear, branched or cyclic and saturated or unsaturated. The hydrogen atoms of the carbon chain can, in addition, be substituted by halogens or halogenated, alkyl, acyl, acyloxy, alkoxycarbonyl or alkoxy radicals having from 2 to 18 carbon atoms.

The hydroxy acids which can be used are in particular glycolic, lactic, malic, tartaric, citric, 2-hydroxyalkanoic, mandelic, and salicylic acids, and their acyl derivatives, such as 5-n-octanoylsalicylic acid, 5-n-dodecanoylsalicylic acid, 5-n-decanoylsalicylic acid, 5-n-octylsalicylic acid, 5-or 4-n-heptyloxysalicylic acid or 2-hydroxy-3-methylbenzoic acid, or alternatively their alkoxy derivatives such as 2-hydroxy-3-methoxybenzoic acid.

The retinoids can be in particular retinoic acid (all trans or 13-cis) and its derivatives, retinol (vitamin A) and its esters such as retinol palmitate, retinol acetate, and retinol propionate, and their salts.

These active agents can be used in particular at concentrations ranging from 0.0001% to 5% by weight based on the total weight of the composition.

When the compositions of the present invention are intended to be injected, they can be provided in the form of solutions containing the excipients commonly used for injections and for example in the form of an isotonic sodium chloride solution.

Other features of the invention will become apparent in the course of the following descriptions of exemplary embodiments which are given for illustration of the invention and are not intended to be limiting thereof.

EXAMPLES

The amounts indicated in the following Examples are percentages by weight. The term "qsp for 100%" means that that ingredient is present in an amount sufficient to make the total amount of all ingredients equal 100% by weight.

Example 1

Care lotion for the face.

Z-Glycine		8%
Antioxidant		0.05%
Preservative		0.3%
Ethanol (solvent)		8%
Water	qsp for	100%

The lotion obtained has an effect on wrinkles during repeated use (twice daily applications for one month).

Example 2

Gel for caring for the face.

Z-Glycine		5%
Hydroxypropylcellulose (Klucel H sold by the company Hercules) (gelling agent)		1%
Preservative		0.3%
Ethanol (solvent)		15%
Antioxidant		0.05%
Water	qsp for	100%

The gel obtained has an effect on wrinkles. It can be applied daily, morning and evening, for one month.

Example 3

Care cream for the face (oil-in-water emulsion).

Flunitrazepam		0.1%
Glyceryl stearate (emulsifier)		2%
Polysorbate 60 (Tween 60 sold by the company ICI) (emulsifier)		1%
Stearic acid		1.4%
Triethanolamine (neutralizing agent)		0.7%
Carbomer (Carbopol 940 sold by the company Goodrich)		0.4%
Karite butter liquid fraction		12%
Perhydrosqualene		12%
Preservative		0.3%
Fragrance		0.5%
Antioxidant		0.05%
Water	qsp for	100%

A white oily cream is obtained which has an effect on wrinkles and fine lines and which can be applied daily.

Example 4

Care cream for the face (oil-in-water emulsion).

Flunitrazepam		0.2%
Glycercyl mono- and distearate		2%
Cetyl alcohol		1.5%
Cetylstearyl alcohol/cetylstearyl alcohol oxyethylenated 33 EO		7%
Dimethylpolysiloxane		1.5%
Liquid paraffin		17.5%
Preservative		0.3%
Fragrance		0.5%
Glycerol		12.5%
Water	qsp for	100%

This application is based on French Patent Application 94-11742 filed on Sep. 30, 1994, which is incorporated herein by reference in its entirety.

Obviously, numerous modifications and variations of the present invention are possible in light of the above teachings. It is therefore to be understood that, within the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.

What is claimed as new and desired to be secured by Letters Patent of the United States is:

1. A method for relaxing or slackening cutaneous tissue, comprising topically applying a cutaneous tissue relaxing or slackening effective amount of at least one agonist substance of at least one receptor associated with at least one chlorine channel present in cutaneous tissue, wherein the agonist substance is selected from the group consisting of serine, taurine, β-alanine, N-(benzyloxycarbonyl)glycine, isoguvacine, isonipecotic acid, 4,5,6,7-tetrahydroisoxazolopyrid-3 (2H)-one, 1,3-dihydro-7-nitro-5-phenyl-2H-1,4-benzodiazepin-2-one, 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one, 5-(2-fluorophenyl)-1,3-dihydro-1-methyl-7-nitro-2H-1,4-benzodiazepin-2-oue, 7-chloro-1,3-dihydro-3-hydroxy-5-phenyl-2H-1,4-benzodiazepin-2-one, 3-hydroxypregnane-11,20-dione, 5,5-diethylbarbituric acid, 5-ethyl-5-(1-methylbutyl)barbituric acid, 5-ethyl-5-phenylbarbituric acid, and their salts.

2. The method of claim 1, wherein said agonist substance activates a glycine-strychnine sensitive receptor and is selected from the group consisting of serine, taurine, β-alanine, and N-(benzyloxycarbonyl)glycine.

3. The method of claim 1, wherein said agonist substance is applied in a composition in an amount ranging from 0.00001 to 20% by weight, based on the total weight of said composition.

4. The method of claim 3, wherein said agonist substance is present in said composition in an amount ranging from 0.01 to 10% by weight, based on the total weight of said composition.

5. The method of claim 3, wherein said composition further comprises at least one of a hydroxy acid and a retinoid.

6. The method of claim 5, wherein said hydroxy acid is selected from the group consisting of α-hydroxy acids and β-hydroxy acids, which can be linear, branched or cyclic and saturated or unsaturated.

7. The method of claim 5, wherein said retinoid is selected from the group consisting of retinoic acid, retinol and retinol esters.

8. A method for lessening wrinkles or fine lines, by relaxing or slackening cutaneous tissue comprising topically applying a wrinkle or fine line lessening effective amount of at least one agonist substance of at least one receptor associated with at least one chlorine channel of at least one cutaneous afferent nerve pathway, wherein the agonist substance is selected from the group consisting of serine, taurine, β-alanine, N-(benzyloxycarbonyl)glycine, isoguvacine, isonipecotic acid, 4,5,6,7-tetrahydroisoxazolo-[5,4-c]pyrid-3(2H)-one, 1,3-dihydro-7-nitro-5-phenyl-2H-1,4-benzodiazepin-2-one, 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one, 5-(2-fluorophenyl)-1,3-dihydro-1-methyl-7-nitro-2H-1,4-benzodiazepin-2-one, 7-chloro-1,3-dihydro-3-hydroxy-5-phenyl-2H-1,4-benzodiazepin-2-one, 3-hydroxypregnane-11,20-dione, 5,5-diethylbarbituric acid, 5-ethyl-5-(1-methylbutyl)barbituric acid, 5-ethyl-5-phenylbarbituric acid, and their salts.

9. The method of claim 8, wherein said agonist substance activates a glycine-strychnine sensitive receptor and is selected from the group consisting of serine, taurine, β-alanine, and N-(benzyloxycarbonyl)glycine.

10. The method of claim 8, wherein said agonist substance is applied in a composition in an amount ranging from 0.00001 to 20% by weight, based on the total weight of said composition.

11. The method of claim 10, wherein said agonist substance is present in said composition in an amount ranging from 0.01 to 10% by weight, based on the total weight of said composition.

12. The method of claim 10, wherein said composition further comprises at least one of a hydroxy acid or a retinoid.

13. The method of claim 12, wherein said hydroxy acid is selected from the group consisting of α-hydroxy acids and β-hydroxy acids, which can be linear, branched or cyclic and saturated or unsaturated.

14. The method of claim 12, wherein said retinoid is selected from the group consisting of retinoic acid, retinol and retinol esters.

15. A method for lessening wrinkles or fine lines, comprising administering by injection a cosmetic or dermatological composition, said composition comprising at least one agonist substance of at least one receptor associated with at least one chlorine channel present in cutaneous tissue, wherein the agonist substance is selected from the group consisting of serine, taurine, β-alanine, N-(benzyloxycarbonyl)glycine, isoguvacine,isonipecotic acid, 4,5,6,7-tetrahydroisoxazolopyrid-3(2H)-one, 1,3-dihydro-7-nitro-5-phenyl-2H-1,4-benzodiazepin-2-one, 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one, 5-(2-fluorophenyl)-1,3-dihydro-1-methyl-7-nitro-2H-1,4-benzodiazepin 2-one, 7-chloro-1,3-dihydro-3-hydroxy-5-phenyl-2H-1,4-benzodiazepin-2-one, 3-hydroxypregnane-11,20-dione, 5,5-diethylbarbituric acid, 5-ethyl-5-(1-methylbutyl)barbituric acid, 5-ethyl-5-phenylbarbituric acid, and their salts.

16. The method of claim 15, wherein said agonist substance activates a glycine-strychnine sensitive receptor and is selected from the group consisting of serine, taurine, β-alanine, and N-(benzyloxycarbonyl)glycine.

17. The method of claim 15, wherein said agonist substance is present in said composition in an amount ranging from 0.00001 to 20% by weight, based on the total weight of said composition.

18. The method of claim 15, wherein said agonist substance is present in said composition in an amount ranging from 0.01 to 10% by weight, based on the total weight of said composition.

19. The method of claim 15, wherein said composition further comprises at least one of a hydroxy acid or a retinoid.

20. The method of claim 19, wherein said hydroxy acid is selected from the group consisting of α-hydroxy acids and

β-hydroxy acids, which can be linear, branched or cyclic and saturated or unsaturated.

21. The method of claim 19, wherein said retinoid is selected from the group consisting of retinoic acid, retinol and retinol esters.

22. A method for relaxing or slackening cutaneous tissue, comprising administering by injection a cosmetic or dermatological composition, said composition comprising at least one agonist substance of at least one receptor associated with at least one chlorine channel of at least one cutaneous afferent nerve pathway, wherein the agonist substance is selected from the group consisting of serine, taurine, β-alanine, N-(benzyloxycarbonyl)glycine, isoguvacine, isonipecotic acid, 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyrid-3(2H)-one, 1,3-dihydro-7-nitro-5-phenyl-2H-1,4-benzodiazepin-2-one, 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one, 5-(2-fluorophenyl)-1,3-dihydro-1-methyl-7-nitro-2H-1,4-benzodiazepin-2-one, 7-chloro-1,3-dihydro-3-hydroxy-5-phenyl-2H-1,4-benzodiazepin-2-one, 3-hydroxypregnane-11,20-dione, 5,5-diethylbarbituric acid, 5-ethyl-5-(1-methylbutyl)barbituric acid, 5-ethyl-5-phenylbarbituric acid, and their salts.

23. The method of claim 22, wherein said agonist substance activates a glycine-strychnine sensitive receptor and is selected from the group consisting of serine, taurine, β-alanine, and N-(benzyloxycarbonyl)glycine.

24. The method of claim 22, wherein said agonist substance is present in said composition in an amount ranging from 0.00001 to 20% by weight, based on the total weight of said composition.

25. The method of claim 22, wherein said agonist substance is present in said composition in an amount ranging from 0.01 to 10% by weight, based on the total weight of said composition.

26. The method of claim 22, wherein said composition further comprises at least one of a hydroxy acid and a retinoid.

27. The method of claim 26, wherein said hydroxy acid is selected from the group consisting of α-hydroxy acids and β-hydroxy acids, which can be linear, branched or cyclic and saturated or unsaturated.

28. The method of claim 26, wherein said retinoid is selected from the group consisting of retinoic acid, retinol and retinol esters.

29. A method for the cosmetic treatment of wrinkles or fine lines in humans, comprising injecting a composition comprising at least one agonist substance of at least one receptor associated with at least one chlorine channel present in cutaneous tissue, wherein the agonist substance is selected from the group consisting of serine, taurine, β-alanine, N-(benzyloxycarbonyl)glycine, isoguvacine, isonipecotic acid, 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyrid-3(2H)-one, 1,3-dihydro-7-nitro-5-phenyl-2H-1,4-benzodiazepin-2-one, 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one, 5-(2-fluorophenyl)-1,3-dihydro-1-methyl-7-nitro-2H-1,4-benzodiazepin-2-one, 7-chloro-1,3-dihydro-3-hydroxy-5-phenyl-2H-1,4-benzodiazepin-2-one, 3-hydroxypregnane-11,20-dione, 5,5-diethylbarbituric acid, 5-ethyl-5-(1-methylbutyl)barbituric acid, 5-ethyl-5-phenylbarbituric acid, and their salts.

30. The method of claim 1, wherein said agonist substance activates a $GABA_A$ receptor and is selected from the group consisting of isoguvacine, isonipecotic acid, 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyrid-3(2H)-one, 1,3-dihydro-7-nitro-5-phenyl-2H-1,4-benzodiazepin-2-one, 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one, 5-(2-fluorophenyl)-1,3-dihydro-1-methyl-7-nitro-2H-1,4-benzodiazepin-2-one, 7-chloro-1,3-dihydro-3-hydroxy-5-phenyl-2H-1,4-benzodiazepin-2-one, 3hydroxypregnane-11,20-dione, 5,5-diethylbarbituric acid, 5-ethyl-5-(1-methylbutyl)barbituric acid, 5-ethyl-5-phenylbarbituric acid, and their salts.

31. The method of claim 8, wherein said agonist substance activates a $GABA_A$ receptor and is selected from the group consisting of isoguvacine, isonipecotic acid, 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyrid-3(2H)-one, 1,3-dihydro-7-nitro-5-phenyl-2H-1,4-benzodiazepin-2-one, 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one, 5-(2-fluorophenyl)-1,3-dihydro-1-methyl-7-nitro-2H-1,4-benzodiazepin-2-one, 7-chloro-1,3-dihydro-3-hydroxy-5-phenyl-2H-1,4-benzodiazepin-2-one, 3-hydroxypregnane-11,20-dione, 5,5-diethylbarbituric acid, 5-ethyl-5-(1-methylbutyl)barbituric acid, 5-ethyl-5-phenylbarbituric acid, and their salts.

32. The method of claim 15, wherein said agonist substance activates a $GABA_A$ receptor and is selected from the group consisting of gamma-aminobutyric acid, isoguvacine, isonipecotic acid, 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyrid-3(2H)-one, 1,3-dihydro-7-nitro-5-phenyl-2H-1,4-benzodiazepin-2-one, 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one, 5-(2-fluorophenyl)-1,3-dihydro-1-methyl-7-nitro-2H-1,4-benzodiazepin-2-one, 7-chloro-1,3-dihydro-3-hydroxy-5-phenyl-2H-1,4-benzodiazepin-2-one, 3-hydroxypregnane-11,20-dione, 5,5-diethylbarbituric acid, 5-ethyl-5-(1-methylbutyl)barbituric acid, 5-ethyl-5-phenylbarbituric acid, and their salts.

33. The method of claim 22, wherein said agonist substance activates a $GABA_A$ receptor and is selected from the group consisting of gamma-aminobutyric acid, isoguvacine, isonipecotic acid 4,5,6,7- tetrahydroisoxazolo pyrid-3(2H)-one, 1,3-dihydro-7-nitro-5-phenyl-2H-1,4-benzodiazepin-2-one, 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one, 5-(2-flurophenyl)-1,3-dihydro-1-methyl-7-nitro-2H-1,4-benzodiazepin-2-one, 7-chloro-1,3-dihydro-3-hydroxy-5-phenyl-2H-1,4-benzodiazepin-2-one, 3-hydroxopregane-11,20-dione, 5,5-diethylbarbituric acid, 5-ethyl-5-(1-methylbutyl)barbituric acid, 5-ethyl-5-phenylbarbituric acid, and their salts.

34. The method of claim 1, wherein said receptor is at least one selected from the group consisting of $GABA_A$ receptors and glycine-strychnine sensitive receptors.

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United States Patent [19]

De Lacharriere et al.

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[54] COMPOSITIONS AND METHODS FOR TREATING WRINKLES AND/OR FINE LINES OF THE SKIN

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[52] **U.S. Cl.** **424/401**; 514/844; 514/846; 514/944

[58] **Field of Search** 424/401; 514/844, 514/846, 944

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[57] **ABSTRACT**

Compositions which contain an agonist substance of one or a number of receptors associated with a chlorine channel are useful for slackering and/or relaxing cutaneous tissue, and in particular for the purpose of treating wrinkles and fine lines of the skin. Such compositions can be administered topically or by injection. Preferred agonists include glycine, serine, taurine, β-alanine, N-(henzyloxycarbonyl)glycine (Z-glycine), gamma-aminobutyric acid (GABA), isoguvacine, isonipecotic acid, 4,5,6,7-tetrahydroisoxazolo [5,4-c]pyrid-3(2H)-one, benzodiazepines, steroids, and barbiturates. The composition can additionally contain a retinoid and/or a hydroxy acid.

25 Claims, No Drawings

COMPOSITIONS AND METHODS FOR TREATING WRINKLES AND/OR FINE LINES OF THE SKIN

This application is a continuation of Ser. No. 08/538,119 filed on Oct. 2, 1995 and now a U.S. Pat. No. 5,869,068.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to the use of substances which are agonists of a receptor associated with a chlorine channel in a cosmetic and/or dermatological composition, in particular for the purpose of treating wrinkles and fine lines of the skin, and to cosmetic and/or dermatological compositions which contain such a substance.

2. Discussion of the Background

Women, and indeed even men, are currently inclined to wish to appear young for as long as possible and consequently are looking to soften the signs of ageing of the skin, which are reflected in particular by wrinkles and fine lines. In this respect, advertising and fashion present products intended to retain a radiant and wrinkle-free skin, these being the signs of young skin, for as long as possible, all the more so since physical appearance has an effect on mental attitude and/or on morale. It is consequently important to feel physically and spiritually young.

Until now, wrinkles and fine lines have been treated using cosmetic products containing active agents which act on the skin, for example by moisturizing it or by improving its cell renewal or alternatively by promoting the synthesis of collagen of which the cutaneous tissue is composed. However, to date, it is not known to act on wrinkles by involving the muscle components present in the skin.

It is known that the platysma muscles of the face are under the control of the motor nerve afferent activity of the facial nerve and that, moreover, the interlobular septa of the hypoderm contain within them fibers which constitute a striated muscle tissue (panniculus carnosus). Moreover, it is also known that a subpopulation of fibroblasts of the dermis, known as myofibroblasts, has characteristics in common with the muscle tissue.

The Applicants have observed, in certain pathological and therapeutic situations, the role played, as regards the wrinkles of the face, by the nerves controlling all this muscle tissue. Thus, in attacks on the facial nerve, in which transmission of the nerve impulse is interrupted and/or weakened, a paralysis of the muscles of the face is witnessed in the area of innervation. This facial paralysis is reflected, among other clinical indications, by an alleviation in, indeed disappearance of, the wrinkles.

On the other hand, in muscle hypercontraction conditions of the face, the Applicants have observed an accentuation in the wrinkles of the face. Moreover, an accentuation in the wrinkles of the face has also been observed in muscle hypertonia conditions of Parkinson's disease and side-effects induced by neuroleptics.

Moreover, it has been shown that botulinus toxin, originally used for treating spasms, could have an effect on muscle spasticity conditions (see A. Blitzer et al., *Arch. Otolaryngol. Head Neck Surg*., vol 119, pages 1018 to 1022 (1993)) and on the wrinkles of the glabella, which are intersuperciliary wrinkles (see J. D. Carruters et al., *J. Dermatol. Surg. Oncol*., vol. 18, pages 17 to 21 (1992)). It is consequently possible, by pharmacological action, to have an effect on the nerve component of wrinkles. Botulinus toxin acts directly at the level of the neuro-muscular junction by blocking the action of acetylcholine on muscular tenseness.

The junction between a nerve and a muscle constitutes the myoneural endplate, before which is found the afferent nerve route known as the motor neuron. Moreover, the cell membranes of each nerve fiber contain many ionic channels, and in particular chlorine channels, capable of allowing the corresponding element to pass through in the ionic form, and, in the case of chlorine channels, in the chloride form. Neuronal receptors are associated with these channels. The neuronal receptors associated with the chlorine channels are in particular receptors for glycine (glycine-strychnine sensitive receptors) and receptors for GABA ($GABA_A$ receptors).

Moreover, it is known that, in the central nervous system, it is possible to decrease the excitability of the neuron by various pharmacological agents which have an effect on the glycine-strychnine sensitive receptors or on the $GABA_A$ receptors of the central nervous system (see W. Sieghart, *Trends in Pharmacological Science*, vol. 131, pages 446 to 450 (December 1992)). Activation of these receptors opens the chlorine channels and leads to the entry of chloride ions, which results in an increase in the chloride ions in the cells of the nerve fiber and thus to hyperpolarization of the neurons, which consequently become less excitable.

On the other hand, in the neuromuscular junction, a decrease in excitability of the motor neuron leads to a lessened stimulation of the muscle fiber, thus causing it to slacken.

However, to date no completely suitable compositions or methods are available for treating wrinkles and/or fine lines of the skin. Thus, there remains a need for methods and compositions effective for treating wrinkles and/or fine lines of the skin.

SUMMARY OF THE INVENTION

Accordingly, it is one object of the present invention to provide novel compositions for treating wrinkles and/or fine lines of the skin.

It is another object of the present invention to provide novel methods for treating wrinkles and/or fine lines of the skin.

These and other objects, which will become apparent during the following detailed description, have been achieved by the inventors' discovery that contractile muscle fibers, which are under the direct control of the neuromotor impulse, play an essential role in the pathogenesis of wrinkles and that suppression of the neuromotor impulse alleviates not only wrinkles but also fine lines and also has a "smoothing" effect on the cutaneous microrelief. It has also been found that cutaneous tissues contain receptors associated with chlorine channels, something which, until now, had not been envisaged. It has thus been found that it is possible to act on these channels in order to slacken or relax these tissues and thus to lessen wrinkles and fine lines.

Until now, a connection between the chlorine channels of nerve fibers of the peripheral cutaneous nervous system and wrinkles had never been established, nor had it been found that it was possible to treat wrinkles by acting on chlorine channels by activation of the receptors which are found in or in the neighborhood of these channels. Substances which can activate the receptors of chlorine channels and thus lead to the entry of chloride into cells are known as agonist substances.

Consequently, the present invention provides topical cosmetic or dermatological compositions, which contain at least

one agonist substance of at least one receptor associated with at least one chlorine channel present in cutaneous tissue except glycine and gamma-aminobutyric acid for relaxing and/or slackening cutaneous tissue.

In another aspect, the present invention provides injectable cosmetic or dermatological compositions, for the purpose of lessening wrinkles and/or fine lines, which contain at least one agonist substance of at least one receptor associated with at least one chlorine channel present in cutaneous tissue for relaxing and/or slackening cutaneous tissue. In this context, the term "injectable" means suitable for injection into tissue, and in particular in wrinkles.

The present invention further provides injectable or topical cosmetic or dermatological compositions, which contain at least one agonist substance of at least one receptor associated with at least one chlorine channel of at least one cutaneous afferent nerve pathway for relaxing and/or slackening cutaneous tissue.

The present invention additionally provides topical cosmetic or dermatological compositions for the purpose of lessening wrinkles and/or fine lines which contain at least one agonist substance of at least one receptor associated with at least one chlorine channel of at least one cutaneous afferent nerve pathway, except glycine and gamma-butyric acid, for relaxing and/or slackening cutaneous tissue.

The compositions containing the agonist according to the present invention can be applied topically or by subcutaneous and/or intradermal and/or "intrawrinkle" injection.

Another aspect of the present invention is a method for the cosmetic treatment of wrinkles and/or fine lines in humans by injecting a composition containing at least one agonist substance of at least one receptor associated with at least one chlorine channel present in cutaneous tissue.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

A number of receptors associated with the chlorine channel exist. They concern in particular glycine-strychnine sensitive receptors and $GABA_A$ receptors, the latter themselves containing a number of subunits comprising the GABA site, the benzodiazepine site, a type of steroid site and the barbiturates site. All the substances which act as agonists of these receptors or sites can be used for slackening or relaxing cutaneous tissues in accordance with the present invention.

For a substance to be recognized as an agonist of a receptor of the chlorine channel, it must exhibit the following two characteristics:

(i) to be able to be bound selectively to at least one of the various receptors associated with the chlorine channel; and

(ii) to show a relaxation effect on a contracted muscle tissue.

The first characteristic, which consists of the possibility of being bound to a receptor associated with a chlorine cbannel, does not make it possible to distinguish an agonist activity from an antagonist activity but it does make it possible to define a potential affinity for the receptor.

The second cbaracteristic makes it possible to select the agonists. The agonist activity of tbe substance under study can be demonstrated by the relaxation effect which it produces on a muscle tissue which has been contracted beforehand by a chlorine channel antagonist substance. Substances known as chlorine channel antagonists can be chosen as such and in particular include the following substances: bicuculline, strychnine, tert-hutylbicyclophosphorothionate and picrotoxin.

Mention may be made, as agonist substances, which can be used in the present invention for activating glycine-strychnine sensitive receptors, of glycine, serine, taurine, β-alanine, and N-(benzyloxycarbonyl)glycine or (Z-glycine).

Mention may be made, as agonist substances, which can be used in the invention for activating $GABA_A$ receptors, of gamma-aminobutyric acid (GABA), isoguvacine, isonipecotic acid, 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyrid-3(2H)-one (THIP), benzodiazepines such as nitrazepam (1,3-dihydro-7-nitro-5-phenyl-2H-1,4-benzodiazepin-2-one), diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one), flunitrazepam (5-(2-fluorophenyl)-1,3-dihydro-1-methyl-7-nitro-2H-1,4-benzodiazepin-2-one) or oxazepam (7-chloro-1,3-dihydro-3-hydroxy-5-phenyl-2H-1,4-benzodiazepin-2-one), certain steroids such as alfaxalone (3-hydroxypregnane-11,20-dione) or barbiturates such as barbital (5,5-diethylbarbituric acid), pentobarbital (5-ethyl-5-(1-methylbutyl)barbituric acid) or phenobarbital (5-ethyl-5-phenylbarbituric acid), and their salts.

It is certainly known to use GABA and glycine in combination with other active agents for combating ageing of the skin but, until now, their action in relaxing and slackening cutaneous tissues for the purpose of treating wrinkles was not known. The generally known actions are inhibition of elastase, the effect on collagen, and cell renewal.

Indeed, it is known in the state of the art to use amino acids as moisturizing agents for the purpose of improving the condition of the skin. In particular, combinations of amino acids such as glycine, taurine or β-alanine in the form of peptide mixtures have been used in cosmetic compositions intended for treating the ageing of the skin. Thus, FR-A-2,546,164 discloses the elastase-inhibiting properties of lipopeptides which prevent deterioration of elastin fibers in the skin, which makes them antiwrinkle active agents. Moreover, U.S. Pat. No. 5,198,465 discloses that amino acids prevent deficiencies in the synthesis of collagen, which consequently makes it possible to prevent ageing of the skin.

In addition, JP-A-05043448 discloses that the combination of GABA and of diisopropylamine facilitates renewal of tbe skin and thus prevents cutaneous ageing.

In the compositions according to the present invention, the agonist of a receptor associated with the chlorine channel is preferably used in an amount ranging from 0.00001 to 20% by weight, based on the total weight of the composition, and in particular in an amount ranging from 0.01 to 10% by weight, based on the total weight of the composition.

The compositions according to the present invention can be provided in all the pharmaceutical dosage forms normally used for a topical or injectable application.

The amounts of the various constituents of the compositions according to the present invention are those conventionally used in the fields under consideration and are appropriate to their pharmaceutical dosage form.

For topical application, the compositions of the present invention comprise a medium compatible with skin. These compositions can be provided in particular in the form of aqueous, alcoholic or aqueous/alcoholic solutions, of gels, of water-in-oil or oil-in-water emulsions having the appearance of a cream or of a gel, of microemulsions or of aerosols or alternatively in the form of vesicular dispersions containing ionic and/or nonionic lipids. These pharmaceutical dosage forms are prepared according to the conventional methods in the fields under consideration.

The present compositions for topical application can constitute in particular a cosmetic or dermatological protection, treatment or care composition for the face, for the neck, for the hands or for the body (for example day creams, night creams, sun creams or oils or body milks), a make-up composition (for example foundation cream), or an artificial tanning composition.

When the composition of the present invention is an emulsion, the proportion of fatty substance which it contains can range from 5% to 80% by weight, and preferably from 5% to 50% by weight, based on the total weight of the composition. The fatty substances and the emulsifiers used in the composition in the emulsion form are chosen from those conventionally used in the cosmetic or dermatological field.

Mention may be made, as fatty substances which can be used in the present invention, of mineral oils (paraffin), vegetable oils (karite butter liquid fraction) and their hydrogenated derivatives, animal oils, synthetic oils (perhydrosqualene), silicone oils (dimethylpolysiloxane), and fluorinated oils. Mention may also be made, as other fatty substances, of fatty alcohols (cetyl alcohol or stearyl alcohol), fatty acids (stearic acid), and waxes.

The emulsifiers can be present in the present compositions in a proportion ranging from 0.3% to 30% by weight, and preferably from 0.5 to 30% by weight, based on the total weight of the composition.

In a conventional way, the cosmetic or dermatological compositions of the present invention can also contain adjuvants which are typical in the corresponding fields, such as hydrophilic or lipophilic gelling agents, preservatives, antioxidants, solvents, fragrances, fillers, screening agents, and colorants. Moreover, these compositions can contain hydrophilic or lipophilic active agents. The amounts of these various adjuvants or active agents are those conventionally used in the cosmetics or dermatological field and, for example, from 0.01% to 20% of the total weight of the composition. These adjuvants or these active agents, depending on their nature, can be introduced into the fatty phase, into the aqueous phase and/or into lipid vesicles.

Mention may especially be made, among the active agents which the compositions of the invention can contain, of active agents having an effect on the treatment of wrinkles or of fine lines and in particular of keratolytic active agents. The term "keratolytic active agent" is understood to mean an active agent having desquamative, exfoliative or scrubbing properties or an active agent capable of softening the corneal layer.

Mention may in particular be made, among these active agents having an effect on the treatment of wrinkles or fine lines which the compositions of the invention can contain, of hydroxy acids and retinoids.

The hydroxy acids can be, for example, a-hydroxy acids or β-hydroxy acids, which can be linear, branched or cyclic and saturated or unsaturated. The hydrogen atoms of the carbon chain can, in addition, be substituted by halogens or halogenated, alkyl, acyl, acyloxy, alkoxycarbonyl or alkoxy radicals having from 2 to 18 carbon atoms.

The hydroxy acids which can be used are in particular glycolic, lactic, malic, tartaric, citric, 2-hydroxyalkanoic, mandelic, and salicylic acids, and their acyl derivatives, such as 5-n-octanoylsalicylic acid, 5-n-dodecanoylsalicylic acid, 5-n-decanoylsalicylic acid, 5-n-octylsalicylic acid, 5-or 4-n-heptyloxysalicylic acid or 2-hydroxy-3-methylbenzoic acid, or alternatively their alkoxy derivatives such as 2-hydroxy-3-methoxybenzoic acid.

The retinoids can be in particular retinoic acid (all trans or 13-cis) and its derivatives, retinol (vitamin A) and its esters such as retinol palmitate, retinol acetate, and retinol propionate, and their salts.

These active agents can be used in particular at concentrations ranging from 0.0001% to 5% by weight based on the total weight of the composition.

When the compositions of the present invention are intended to be injected, they can be provided in the form of solutions containing the excipients commonly used for injections and for example in the form of an isotonic sodium chloride solution.

Other features of the invention will become apparent in the course of the following descriptions of exemplary embodiments which are given for illustration of the invention and are not intended to be limiting thereof.

EXAMPLES

The amounts indicated in the following Examples are percentages by weight. The term "qsp for 100%" means that ingredient is present in an amount sufficient to make the total amount of all ingredients equal 100% by weight.

Example 1

Care lotion for the face.

Z-Glycine	8%
Antioxidant	0.05%
Preservative	0.3%
Ethanol (solvent)	8%
Water	qsp for 100%

The lotion obtained has an effect on wrinkles during repeated use (twice daily application for one month).

Example 2

Gel for caring for the face.

Z-Glycine	5%
Hydroxypropylcellulose (Klucel H sold by the company Hercules) (gelling agent)	1%
Preservative	0.3%
Ethanol (solvent)	15%
Antioxidant	0.05%
Water	gsp for 100%

The gel obtained has an effect on wrinkles. It can be applied daily, morning and evening, for one month.

Example 3

Care cream for the face (oil-in-water emulsion).

Flunitrazepam	0.1%
Glyceryl stearate (emulsifier)	2%
Polysorbate 60 (Tween 60 sold by the company ICI) (emulsifier)	1%
Stearic acid	1.4%
Triethanolamine (neutralizing agent)	0.7%
Carbomer (Carbopol 940 sold by the company Goodrich)	0.4%
Karite butter liquid fraction	12%
Perhydrosqualene	12%
Preservative	0.3%

-continued

Fragrance	0.5%
Antioxidant	0.05%
Water	gsp for 100%

A white oily cream is obtained which has an effect on wrinkles and line lines and which can be applied daily.

Example 4

Care cream for the face (oil-in-water emulsion).

Flunitrazepam	0.2%
Glycercyl mono- and distearate	2%
Cetyl alcohol	1.5%
Cetylstearyl alcohol/cetylstearyl alcohol oxyethylenated 33 EO	7%
Dimethylpolysiloxane	1.5%
Liquid paraffin	17.5%
Preservative	0.3%
Fragrance	0.5%
Glycerol	12.5%
Water	gsp for 100%

This application is based on French Patent Application 94-11742 filed on Sep. 30, 1994, which is incorporated herein by reference in its entirety.

Obviously, numerous modifications and variations of the present invention are possible in light of the above teachings. It is therefore to be understood that, within the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.

What is claimed as new and desired to be secured by Letters Patent of the United States is:

1. A method for relaxing or slackening cutaneous tissue, comprising topically applying to cutaneous tissue a cutaneous tissue relaxing or slackening effective amount of at least one agonist substance of at least one receptor associated with at least one chlorine channel present in cutaneous tissue, wherein said agonist substance is a benzodiazepine, a steroid or a barbiturate.

2. The method of claim 1, wherein said agonist substance is applied in a composition in an amount ranging from 0.00001 to 20% by weight, based on the total weight of said composition.

3. The method of claim 2, wherein said agonist substance is applied in a composition in an amount ranging from 0.01 to 10% by weight, based on the total weight of said composition.

4. The method of claim 3, wherein said composition further comprises at least one of a hydroxy acid and a retinoid.

5. The method of claim 4, wherein said hydroxy acid is selected from the group consisting of a-hydroxy acids and β-hydroxy acids, which can be linear, branched or cyclic and saturated or unsaturated.

6. The method of claim 4 wherein said retinoid is selected from the group consisting of retinoic acid, retinol and retinol esters.

7. A method for lessening wrinkles or fine lines, by relaxing or slackening cutaneous tissue comprising topically applying a wrinkle or fine line lessening effective amount of at least one agonist substance of at least one receptor associated with at least one chlorine channel of at least one cutaneous afferent nerve pathway, with the proviso that said agonist is a benzodiazepine, a steroid or a barbiturate.

8. The method of claim 7, wherein said agonist substance is applied in a composition in an amount ranging from 0.00001 to 20% by weight, based on the total weight of said composition.

9. The method of claim 8, wherein said agonist substance is present in said composition in an amount ranging from 0.01 to 10% by weight, based on the total weight of said composition.

10. The method of claim 8, wherein said composition further comprises at least one of a hydroxy acid and a retinoid.

11. The method of claim 10, wherein said hydroxy acid is selected from the group consisting of a-hydroxy acids and β-hydroxy acids, which can be linear, branched or cyclic and saturated or unsaturated.

12. The method of claim 10, wherein said retinoid is selected from the group consisting of retinoic acid, retinol and retinol esters.

13. A method for lessening wrinkles or fine lines, comprising administering by injection a cosmetic or dermatological composition, said composition comprising at least one agonist substance of at least one receptor associated with at least one chlorine channel present in cutaneous tissue wherein said agonist substance is a benzodiazepine, steroid or barbiturate.

14. The method of claim 13, wherein said agonist substance is present in said composition in an amount ranging from 0.00001 to 20% by weight, based on the total weight of said composition.

15. The method of claim 13, wherein said agonist substance is present in said composition in an amount ranging from 0.01 to 10% by weight, based on the total weight of said composition.

16. The method of claim 13, wherein said composition further comprises at least one of a hydroxy acid and a retinoid.

17. The method of claim 16, wherein said hydroxy acid is selected from the group consisting of a-hydroxy acids and β-hydroxy acids, which can be linear, branched or cyclic and saturated or unsaturated.

18. The method of claim 16, wherein said retinoid is selected from the group consisting of retinoic acid, retinol and retinol esters.

19. A method for relaxing or slackening cutaneous tissue, comprising administering by injection a cosmetic or dermatological composition, said composition comprising at least one agonist substance of at least one receptor associated with at least one chlorine channel of at least one cutaneous afferent nerve pathway wherein said agonist substance is a benzodiazepine, steroid or barbiturate.

20. The method of claim 19, wherein said agonist substance is present in said composition in an amount ranging from 0.00001 to 20% by weight, based on the total weight of said composition.

21. The method of claim 19, wherein said agonist substance is present in said composition in an amount ranging from 0.01 to 10% by weight, based on the total weight of said composition.

22. The method of claim 19, wherein said composition further comprises at least one of a hydroxy acid and a retinoid.

23. The method of claim 22, wherein said hydroxy acid is selected from the group consisting of a-hydroxy acids and β-hydroxy acids, which can be linear, branched or cyclic and saturated or unsaturated.

24. The method of claim 22, wherein said retinoid is selected from the group consisting of retinoic acid, retinol and retinol esters.

25. A method for the cosmetic treatment of wrinkles or fine lines in humans, comprising injecting a composition comprising at least one agonist substance of at least one receptor associated with at least one chlorine channel present in cutaneous tissue wherein said agonist substance is a benzodiazepine, steroid or barbiturate.

* * * * *

EXHIBIT N

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Proximate Composition, Free Amino Acids and Peptides Contents in Commercial Chicken and Other Meat Essences

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ABSTRACT

Ten commercial meat essenees of chicken, beef, clam and eel contained the moisture ranged from 91.1 to 97.5%, protein from 0.6 to 7.8%, and ash from 0.3 to 1.7%. Freshwater clam and hard clam essences had a much higher level of glycogen than other products. The total amount of free amino acids (FAA) in six chicken essences varied from 150 to 725 mg/100 g. Taurine was the dominant FAA and accounted for 16~30% of the total FAA. Though beef essence had the highest level of total FAA among ten products, its taurine was accounted for only about 2%. Taurine in freshwater clam essence was also very low, but a high level of ornithine was found. Anserine in six chicken essences, ranging from 36 to 437 mg/100 g, was higher than carnosine, ranging from 8 to 162 mg/100 g. In beef essence, however, anserine was far less than carnosine. These dipeptides in both clam essences were not detectable, and only a small amount of carnosine was present in eel essence. Another dipeptide, balenine, was found only in a chicken essence. The amount of low-molecular-weight peptides in chicken essences, ranged from 1,187 to 3,296 mg/100 g, was more than the level of FAA in each product. Both clam essences contained a much lower amount of small peptides than other products. Consequently, hard clam essence was the only product with lower level of peptides than its FAA.

Key words: chicken essence, meat essence, proximate composition, free amino acid, anserine, carnosine, taurine, peptide

INTRODUCTION

In Southeast Asia region, particularly in Chinese communities, chicken essence is consumed as a traditional health food for several ailments, including the use as a nutritional supplement for sickness, enhancement of mental efficiency, and recovery from mental fatigue[1]. Studies on the effects of chicken essence consumption in human subjects have shown that chicken essence increased the metabolic rate in healthy volunteers[2,3] and increased the restoration of serum Fe after blood donation in females[4]. The consumption of chicken essence caused an increase in brain 5-hydroxytryptamine (5-HT) in the cerebrospinal fluid of rat and might lead to the activation of 5-HT-dependent physiological process like sleep improvement, mood elevation and regular of circadian rhythm[5]. Recent studies reported that chicken essence could markedly suppress the elevation of blood pressure, cardiovascular hypertrophy and renal damage in rats exhibiting symptoms of hypertension[6,7].

Extracts of meat tissues contain free amino acids (FAA), peptides and other non-protein nitrogenous compounds which are not incorporated in proteins. These compounds have been implicated as being responsible for the characteristic taste of food[8,9]. Some specific compounds were proposed as the active components for biological activities of chicken essence[3,6,10], of which taurine (2-aminoethanesulfonic acid), anserine (β-alanyl-1-methylhistidine), carnosine (β-alanylhistidine) and certain small peptides play important roles in physiological functions within the human body. Taurine is very widespread in the animal kingdom and usually occurs in rather high quantities. It is an essential growth factor followed recognition of the role in bile acid synthesis and in prevention of certain pathological problems. Its accumulation is necessary for functional regulation of the eyes, heart, muscle, brain and central nervous system[11-13]. Carnosine and anserine were the major dipeptides in the skeletal muscle tissue of most vertebrates[14-18]. These dipeptides play important roles in physiological functions, such as a potent intracellular pH-buffer[17,18], inhibition of oxidative reactions[16,18,19], activation of enzyme activity[20,21] and neurotransmitter function[22].

In addition to chicken essence, the essences of beef, clam and eel also have been available in the markets of Taiwan as nutritional supplements. Although considerable efforts have been devoted to analyzing non-protein nitrogenous compounds from raw chicken meat, beef, clam and eel, information on these compounds in meat essences was limited. The present study was therefore undertaken to establish basic data of proximate composition, FAA, dipeptides and other small peptides in chicken, beef, clam and eel essences available in the markets.

MATERIALS AND METHODS

I. *Materials*

Commercial meat essences, including beef, freshwater clam, hard clam, eel and six types of chicken essence, were purchased from June 1999 to December 2000 from markets in Keelung and Taipei, Taiwan. According to the labeling,

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they are all natural extracts without additional ingredients like Chinese medicines or plant extracts. Three sets of samples were collected for each product. At least 3 cans from each group were combined and homogenized for chemical analyses.

II. *Extraction of Free Amino Acids and Peptides*

Extracts of FAA and peptides were prepared according to the method of Konosu *et al.*[23]. A 5 g sample was homogenized for 2 mins in 20 mL of 7% cold trichloroacetic acid (TCA) using a Polytron homogenizer. The homogenate was centrifuged at 4,000 g (4°C) for 20 mins. The precipitate was extracted twice with TCA by the same procedure. The supernatants were combined and made up to 100 mL with TCA. 40 mL of TCA-extracted supernatant was mixed with an equal amount of ether to remove the TCA. This procedure was repeated successively five times. The aqueous solution was evaporated to dryness in vacuum evaporator at a temperature below 40°C. The dried matter was diluted with water and made up to 25 mL for FAA and peptides analyses. All analyses were done in three replicates.

III. *Chemical Analyses*

(I) *Proximate Composition*

Moisture, crude protein and ash were determined according to the AOAC[24] method. Crude protein was estimated from the total amount of nitrogen multiplied by 6.25.

(II) *Glycogen*

The glycogen content was determined with minor modification to the method described by Carroll *et al.*[25]. A 1-mL portion of the TCA extract was added to 1 mL of 95% alcohol in test tubes. The mixture was incubated at 37°C for 3 hrs, and then centrifuged at 4,000 g for 15 mins. The precipitate was added to 5 mL of anthrone and the tightly closed tubes was heated in the boiling bath for 15 mins, and then cooled. The absorbance of the resulting solution was read at 620 nm. Glycogen concentration was expressed as glucose concentration. It was estimated from the standard curve multiplied by 0.9.

(III) *Free Amino Acids, Anserine and Carnosine*

The FAA and dipeptides, carnosine and anserine, were separated by ion exchange chromatography and analyzed by a Hitachi L-8500 high-speed amino acid analyzer with a Hitachi 2622 SC packed column (4.6 mm × 60 mm). The buffers used were the standard lithium citrate buffers. Postcolumn derivatization with ninhydrin yielded amino acid derivatives which were measured the absorbance at 570 and 440 nm. Analytical conditions and procedure were performed according to the manual provided by the manufacturer (Hitachi, Ltd., Tokyo, Japan). FAA levels were estimated on the basis of peak areas of known standard concentrations (Wako, Ltd., Osaka, Japan) using a Hitachi D-2850 Chromato data processor.

(IV) *Amino Acids of Low-Molecular-Weight Peptides*

Amino acids were measured before and after hydrolysis of the TCA extracts with 6N HCl at 110°C for 24 hrs in an evacuated sealed tube. Amino acids obtained without prehydrolysis of HCl were free amino acids as mentioned above. The difference between the value of amino acids with HCl hydrolysis and free amino acids were referred to as the constituent amino acids of low-molecular-weight peptides[8,26].

RESULTS AND DISCUSSION

I. *Proximate Composition and Glycogen Contents*

The proximate composition and glycogen contents of ten commercial essences of chicken, beef, eel, hard clam and freshwater clam are shown in Table 1. Fat was not measured in this study because it was removed almost completely during the processing of meat essences. The moisture content of the ten products ranged from 91.1 to 97.5%, protein from 0.6 to 7.8%, and ash from 0.3 to 1.7%. On a dry weight basis, protein was the predominant proximate composition in these products, followed by ash. The $CaCl_2$, usually used

Table 1. Proximate composition (%)[a] and glycogen contents (mg/100 g)[a] of commercial meat essences

	Moisture	Crude protein	Ash	Glycogen
Chicken A[b]	91.9 ± 0.1	7.8 ± 0.3	0.7 ± 0.1	229 ± 135
B	95.6 ± 0.1	2.7 ± 0.1	0.9 ± 0.1	201 ± 83
C	91.1 ± 0.1	7.0 ± 0.3	1.7 ± 0.2	212 ± 13
D	94.8 ± 0.3	3.6 ± 0.1	1.3 ± 0.2	113 ± 98
E	92.1 ± 0.0	6.9 ± 0.0	1.1 ± 0.1	264 ± 174
F	93.3 ± 0.1	6.7 ± 0.0	0.5 ± 0.1	222 ± 192
Beef	92.0 ± 0.0	7.1 ± 0.1	0.6 ± 0.0	214 ± 176
Hard clam	96.3 ± 0.1	4.5 ± 0.0	0.4 ± 0.1	870 ± 163
Freshwater clam	97.5 ± 0.1	0.6 ± 0.0	0.3 ± 0.0	1234 ± 49
Eel	89.6 ± 0.2	4.5 ± 0.1	0.4 ± 0.1	346 ± 151

a: Expressed as mean ± S.D. of triplicate. b: A~F: different brands of chicken essences.

in the extraction procedure of chicken essence, may result in a high content of ash present in chicken essences. An extraordinary feature for freshwater clam and hard clam essences was that they contained a much higher amount of glycogen than other products. Shellfish is rich in glycogen, which plays an important role in energetic and metabolic supply of gametogenesis in many bivalves[27]. Although glycogen is tasteless, it improves the characteristic flavor of shellfish[28].

II. *Free Amino Acids Contents*

Table 2 shows the constituents of FAA (expressed as mg/100 g wet weight) in chicken essences. Total FAA of the six chicken essences ranged from 150 to 725 mg/100 g, of which Products C and E characteristically possessed a much higher amount. Even on a dry weight basis, the total FAA in both products was 2 ~ 4 times higher than others. This finding indicates that the processing method of commercial chicken essence is markedly different from each other, and Products C and E may not be the natural extracts of chicken meat.

Each product's FAA level was also different. The predominant FAA was taurine, and the levels in chicken essences ranged from 16 to 30% of the total FAA. Several critical reviews[11-13] revealed that taurine in mammalian systems was capable of membrane stabilization, bile salt formation, growth modulation, osmoregulation, glycolysis and glycogenesis stimulation, calcium homeostasis maintenance, and antioxidation. Taurine was an essential amino acid for cats, and an inadequate supply could lead to blindness[29]. With these in consideration, taurine is an important component for chicken essence as a health food. Other abundant FAA in chicken essences included glutamic acid, glycine and alanine. The relative proportion of glutamic acid to the total FAA in products B, C and F were 22, 15 and 18%, respectively. Glutamic acid, often used in processed foods, made an important contribution to umami tastes, similar to that of monosodium glutamate[9]. Glycine, the dominant FAA in product E, accounted for 56% of the total FAA. The value was much higher than those in other chicken essences. It seems that an added glycine results in the high quantity of glycine in Product E. This finding provides another evidence showing that Product E is not a natural extract of chicken meat as mentioned above. Glycine is responsible for the sweet taste[8,9]. Therefore, its addition to the chicken essence may enhance the sweetness of the product.

The constituents of FAA in commercial essences of beef, hard clam, freshwater clam and eel are shown in Table 3. Beef essence had the highest concentration of total FAA among the ten products. Aspartic acid, glutamic acid, leucine, lysine and arginine were the major FAA, which together accounted for 51% of the total FAA. However, taurine accounted for only about 2%. In contrast to beef essence, freshwater clam essence contained the lowest amount of FAA among all products. The dominant FAA in freshwater calm essence included alanine, arginine, glutamic acid and ornithine. The FAA composition profile of freshwater clam essence was similar to that of raw clam *Corbicula fluminea*[30]. Freshwater clam essence characteristically contained a high level of ornithin. However, its taurine was very low. The accumulation of taurine in animal has been shown to function as the main cellular osmo-effector[8,27]. Freshwater clam grows in an environment with low salinity; therefore, they may need lower taurine for osmoregulation reagent. The FAA composition profile of hard clam essence was similar to that of raw clam *Meretrix lusoria*[31]. Alanine, taurine, lysine, glycine, glutamic acid and arginine were the major FAA. The total FAA in hard clam

Table 2. Free amino acids contents (mg/100 g)[a] in chicken essences

	A[b]	B	C	D	E	F
Phosphoserine	1.9 ± 1.3	2.0 ± 1.4	3.9 ± 1.7	2.8 ± 1.6	4.9 ± 2.9	2.1 ± 1.7
Taurine	45.0 ± 10.7	24.9 ± 12.6	186.2 ± 29.4	53.0 ± 12.9	111.1 ± 27.5	50.3 ± 16.6
Aspartic acid	5.4 ± 2.3	6.5 ± 2.1	34.1 ± 6.7	4.4 ± 1.0	13.4 ± 3.6	8.5 ± 3.8
Threonine	3.7 ± 1.8	4.1 ± 1.8	28.1 ± 5.1	4.0 ± 1.4	10.9 ± 2.5	5.0 ± 1.3
Serine	7.4 ± 2.5	7.0 ± 2.9	43.7 ± 7.2	3.4 ± 1.1	18.5 ± 4.2	9.8 ± 1.6
Glutamic acid	13.0 ± 4.4	35.5 ± 16.3	107.4 ± 17.5	23.1 ± 5.6	31.2 ± 7.6	36.7 ± 8.9
Glycine	8.4 ± 2.4	14.7 ± 5.8	47.5 ± 4.7	15.3 ± 4.7	383.6 ± 91.0	8.6 ± 2.5
Alanine	10.2 ± 3.3	11.1 ± 4.5	59.4 ± 6.6	30.2 ± 8.7	22.1 ± 5.7	11.5 ± 2.9
Valine	8.8 ± 4.9	10.3 ± 3.9	23.0 ± 5.2	15.5 ± 8.6	12.5 ± 7.1	10.9 ± 7.2
Methionine	4.7 ± 1.4	5.0 ± 0.6	11.0 ± 1.6	5.8 ± 1.9	6.5 ± 1.1	5.6 ± 2.2
Isoleucine	2.4 ± 0.5	3.2 ± 2.1	12.5 ± 2.9	5.6 ± 2.4	5.9 ± 1.1	3.5 ± 2.0
Leucine	4.3 ± 1.1	4.9 ± 3.3	23.3 ± 6.0	10.0 ± 3.4	8.8 ± 1.7	8.0 ± 5.0
Tyrosine	4.3 ± 1.1	5.1 ± 2.5	18.1 ± 3.7	6.5 ± 1.6	7.5 ± 1.2	5.0 ± 1.4
Phenylalanine	4.9 ± 3.9	5.2 ± 2.5	14.5 ± 5.1	6.1 ± 3.9	4.3 ± 2.2	6.8 ± 4.9
β-Alanine	1.7 ± 0.3	1.1 ± 0.3	9.5 ± 0.7	3.8 ± 1.2	5.5 ± 1.6	2.7 ± 0.9
Ornithine	—[c]	0.9 ± 0.1	1.8 ± 1.4	2.6 ± 1.3	1.4 ± 0.4	0.6 ± 0.3
Lysine	5.0 ± 2.9	5.9 ± 2.5	36.8 ± 8.3	11.2 ± 6.6	12.0 ± 3.5	6.9 ± 2.8
Histidine	2.4 ± 1.4	1.1 ± 0.4	10.0 ± 2.3	6.9 ± 6.7	3.8 ± 1.2	3.1 ± 1.0
Arginine	7.6 ± 5.3	3.9 ± 2.3	28.1 ± 7.4	6.6 ± 2.1	11.6 ± 0.9	7.0 ± 2.5
Proline	6.7 ± 5.2	7.0 ± 1.2	23.4 ± 4.7	4.6 ± 4.0	8.7 ± 2.3	5.1 ± 4.4
Total	150.1 ± 35.0	159.5 ± 69.1	724.7 ± 100.8	221.4 ± 80.6	686.9 ± 171.0	199.1 ± 74.8

a: Expressed as mean ± S.D. of triplicate. b: A~F: different brands of chicken essences. c: Not detectable.

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Table 3. Free amino acids contents (mg/100 g)[a] in beef, clam and eel essences

	Beef	Hard clam	Freshwater clam	Eel
Phosphoserine	6.2 ± 2.1	5.8 ± 0.6	1.3 ± 0.3	9.3 ± 4.0
Taurine	24.5 ± 3.6	77.0 ± 16.1	0.8 ± 0.5	29.4 ± 13.5
Aspartic acid	135.3 ± 26.1	10.7 ± 0.2	1.9 ± 0.3	12.2 ± 6.2
Threonine	55.5 ± 10.4	7.3 ± 0.9	1.5 ± 0.3	12.3 ± 5.5
Serine	60.4 ± 11.2	8.1 ± 0.2	1.4 ± 0.2	8.2 ± 3.9
Glutamic acid	109.8 ± 19.5	44.7 ± 5.4	6.0 ± 0.9	19.4 ± 8.5
Glycine	23.6 ± 3.9	51.7 ± 4.1	2.0 ± 0.4	8.4 ± 3.7
Alanine	86.0 ± 17.9	119.7 ± 2.3	9.0 ± 1.1	21.6 ± 9.7
Valine	69.9 ± 11.0	10.4 ± 2.1	1.8 ± 0.2	19.4 ± 8.2
Methionine	47.2 ± 9.3	7.1 ± 4.3	1.6 ± 0.1	19.0 ± 8.9
Isoleucine	71.1 ± 20.3	8.1 ± 3.7	1.1 ± 0.2	14.5 ± 6.4
Leucine	190.1 ± 38.2	12.1 ± 2.1	2.6 ± 0.4	44.7 ± 19.6
Tyrosine	56.6 ± 11.0	9.6 ± 2.2	3.1 ± 0.4	12.3 ± 5.5
Phenylalanine	78.3 ± 16.3	8.9 ± 2.4	0.9 ± 0.2	27.9 ± 11.8
β-Alanine	7.6 ± 5.8	2.5 ± 0.9	1.6 ± 0.5	13.4 ± 7.4
Ornithine	6.3 ± 2.3	1.3 ± 0.4	5.2 ± 1.0	1.7 ± 1.2
Lysine	126.1 ± 21.4	64.9 ± 22.5	3.4 ± 0.7	35.3 ± 15.1
Histidine	29.5 ± 5.0	2.9 ± 0.2	0.5 ± 0.1	12.4 ± 6.3
Arginine	107.4 ± 28.7	34.8 ± 6.6	7.3 ± 1.4	23.3 ± 10.8
Proline	10.8 ± 1.6	7.0 ± 1.2	—[b]	8.6 ± 3.7
Total	1305.0 ± 266.8	494.9 ± 79.0	62.3 ± 12.7	353.8 ± 154.4

a: Expressed as mean ± S.D. of triplicate. b: Not detectable.

essence was eight times higher than that in freshwater clam essence. In addition, the former contained higher taurine, but lower ornithine. Hard clam is a marine shellfish, so it needs more FAA such as taurine for osmo-regulation function. Alanine was the predominant FAA in the essences of both clams. This FAA has been recognized as a taste-active compound for shellfish[8,9]. The FAA pattern of eel essence (Table 3) was different from that of raw eel *Anguilla japonica*[32]. Leucine, lysine, taurine, phenylalanine and arginine were the major FAA in the eel essence. However, the edible meat of raw eel was rich in taurine, glycine, lysine and alanine[32]. Taurine accounted for 30% of the total FAA in eel meat, but only 8% in the essence.

The results indicate that the meat essences can be inferred as a rich resource of taurine, which has been considered benefitial to human health. The glutamic acid, glycine, alanine and arginine are recognized as the taste-active components of various foods[8,9]. The high concentration of these FAA present in the meat essences consequently contributes to the taste of the products.

III. *Anserine and Carnosine Contents*

Table 4 shows the amounts of dipeptides, carnosine and anserine, of ten commercial meat essences. The dipeptide levels were different for each product. Carnosine contents in six chicken essences ranged from 8 to 162 mg/100 g, and anserine contents from 36 to 437 mg/100 g. Product B had much lower levels of anserine and carnosine than other chicken essences. In contrast, Product C not only contained the highest level of FAA (Table 2), but also possessed the highest amount of carnosine and anserine among six chicken essences. Anserine content was found higher than carnosine

Table 4. Carnosine and anserine contents (mg/100 g)[a] of commercial meat essences

	Carnosine	Anserine
Chicken A[b]	83.1 ± 41.5	161.3 ± 61.3
B	7.8 ± 3.9	35.9 ± 16.1
C	161.9 ± 66.0	436.7 ± 118.9
D	138.2 ± 77.6	313.6 ± 42.8
E	129.6 ± 15.8	275.0 ± 46.0
F	101.1 ± 41.0	169.9 ± 31.1
Beef	127.4 ± 43.9	13.5 ± 3.9
Hard clam	—[c]	—
Freshwater clam	—	—
Eel	12.1 ± 9.1	—

a: Expressed as mean ± S.D. of triplicate.
b: A~F: different brands of chicken essences.
c: Not detectable.

content in chicken essence. However, the former was far less than the latter in beef essence. These two dipeptides in both clam essences were not detectable, and only a small amount of carnosine was found in eel essence.

The meat extracts of chicken, turkey and beef had high concentrations of anserine and carnosine[10,14,16]. Commercial chicken essence B particularly contained very low levels of anserine and carnosine, indicating this product might be made from chicken bones, instead of edible meat. Copious amounts of these dipeptides were found in the white muscle of marine pelagic fish, such as tuna, skipjack, salmon, and eel, but they were not distributed in mollusks and crustaceans[8,15,18]. Carnosine was abundant in the eel meat[8,18,32] but very little in eel essence as seen in Table 4. The cause of this result needs further investigation.

Histidine-containing dipeptide, carnosine and anserine, have many physiological functions for animals. It has been

recognized for a long time as a potent intracellular pH-buffer[8,17,18]. An antioxidative activity has recently attracted considerable attention because of effective prevention of oxidation of foods and disease like eye disease related to lifestyle[16,19,33,34]. A proposed mechanism of antioxidative activities of carnosine is ascribed to their oxidation based on the inhibition of free radical reaction[16,19]. However, the detailed mechanisms are still unclear. In addition, the biological roles of these two dipeptides have been postulated to include the control of enzyme activity[20,21] and neurotransmitter function[22]. Hence, the roles of dipeptides as nutritional ingredients and as endogenous antioxidants in meat essences cannot be underestimated.

IV. *Constituted Amino Acids of Low-Molecular-Weight Peptides*

Since FAA increased after hydrolysis of the TCA extracts of meat essences, it was evident that different kinds of low-molecular-weight peptides were present in the extracts. The constituent amino acids of low-molecular-weight peptides in the six commercial chicken essences are shown in Table 5. The total amount of the peptides varied from 1,187 to 3,296 mg/100 g, which was more than FAA present in each product. This finding indicates that commercial chicken essences contained more low-molecular-weight peptides than FAA in the TCA extract. Product E contained the highest amount of peptides among all products. On the dry weight basis, the total FAA level in this product was also the highest. This finding supports that Product E may not be the natural extract of chicken meat as mentioned above.

Glycine, proline, alanine, glutamic acid and aspartic acid were the major constituent amino acids of small peptides in chicken essences. Taurine, the predominant FAA (Table 2), was not detectable or detectable only in a trace amount (Table 5). Apparently, taurine was not a constituent of small peptides but existed as a free form in the extract. Carnosine and anserine were included in low-molecular-weight peptides. After hydrolysis of the TCA extract using high concentration of HCl, the constituted amino acids of the dipeptides, including β-alanine, histidine and 1-methyl-histidine were detected in high levels as shown in Table 5. Product C had higher amounts of anserine and carnosine than those of Product B (Table 4). Consequently, the former contained more β-alanine, histidine and 1-methylhistidine than the latter (Table 5). Another dipeptide, balenine, was not measured in this study. The whales particularly accumulated more than 1,500 mg/100 g[9,15,18] of balenine, which dipeptide consists of β-alanine and 3-methylhistidine. Product D was the only product which characteristically contained 89 mg/100 g of 3-methylhistidine. It is proposed Product D may contain balenine.

Table 6 shows the amino acid profiles of the peptides in beef, hard clam, freshwater clam and eel essences. Beef and eel essences had a much higher level of the total peptides than both clam essences. Glutamic acid, aspartic acid, glycine, lysine, proline, alanine and arginine were the major constituent compounds in beef and eel essences. According to the labeling of beef essence, the protease hydrolyzate was used as the ingredient in this product. In addition to peptides, beef essence had the highest concentration of total FAA among ten products (Table 3). The results of this study supported that enzymatic processing was applied in the processing of beef essence, resulting in high levels of FAA and

Table 5. Constituted amino acids of peptides contents (mg/100 g)^a in chicken essences

	A^b	B	C	D	E	F
Phosphoserine	0.9 ± 0.9	1.4 ± 2.6	0.1 ± 1.9	1.2 ± 1.4	–	1.3 ± 3.1
Taurine	–^{c}	7.5 ± 0.2	–	–	–	0.1 ± 23.7
Aspartic acid	125.4 ± 10.3	89.5 ± 19.3	108.7 ± 10.5	71.1 ± 23.9	222.1 ± 20.4	165.9 ± 40.6
Threonine	44.0 ± 3.2	33.2 ± 6.4	36.5 ± 5.9	26.7 ± 13.3	80.7 ± 10.2	56.0 ± 14.9
Serine	54.2 ± 4.6	46.5 ± 8.1	44.9 ± 10.7	29.8 ± 10.8	110.4 ± 15.2	72.7 ± 20.8
Glutamic acid	148.3 ± 95.5	116.4 ± 78.3	167.6 ± 155.3	107.3 ± 109.3	304.3 ± 202.9	193.3 ± 167.2
Glycine	376.7 ± 29.6	280.6 ± 68.3	307.8 ± 18.2	115.7 ± 32.1	735.2 ± 148.6	507.3 ± 105.2
Alanine	153.8 ± 14.9	115.3 ± 26.3	119.6 ± 12.7	57.4 ± 33.3	262.2 ± 30.0	205.6 ± 50.8
Valine	26.4 ± 7.3	18.3 ± 10.2	26.7 ± 7.0	17.0 ± 17.2	60.9 ± 8.7	32.9 ± 18.7
Methionine	15.5 ± 6.1	9.3 ± 4.6	15.7 ± 3.9	2.7 ± 1.5	36.8 ± 2.0	23.3 ± 4.7
Isoleucine	29.3 ± 2.1	20.5 ± 6.5	26.7 ± 5.9	18.0 ± 5.3	56.0 ± 8.2	36.6 ± 3.2
Leucine	65.0 ± 5.4	49.0 ± 12.3	52.1 ± 8.2	40.7 ± 17.9	130.7 ± 7.9	82.9 ± 21.9
Tyrosine	16.3 ± 1.1	15.2 ± 4.4	14.0 ± 6.4	16.9 ± 5.5	38.5 ± 2.6	24.1 ± 8.1
Phenylalanine	34.1 ± 3.9	28.0 ± 5.8	24.4 ± 6.2	17.8 ± 7.9	71.0 ± 4.6	47.8 ± 14.5
β-Alanine	48.5 ± 12.0	17.8 ± 3.5	37.0 ± 24.2	154.6 ± 49.7	129.3 ± 14.2	64.2 ± 15.0
Ornithine	3.1 ± 2.5	3.6 ± 1.4	5.0 ± 0.9	5.2 ± 0.9	6.5 ± 3.2	8.2 ± 11.8
Lysine	80.6 ± 6.2	62.9 ± 11.8	79.2 ± 11.5	56.0 ± 23.7	166.1 ± 13.7	109.5 ± 24.1
1-Methylhistidine	72.3 ± 21.4	28.5 ± 4.1	218.6 ± 41.7	61.1 ± 21.1	199.7 ± 24.1	96.5 ± 21.2
Histidine	54.0 ± 9.4	21.9 ± 2.2	96.7 ± 13.2	199.8 ± 74.6	115.4 ± 12.3	77.5 ± 18.0
3-Methylhistidine	–	–	–	88.7 ± 27.1	–	–
Arginine	128.5 ± 12.3	101.2 ± 23.3	99.4 ± 10.3	43.3 ± 13.1	232.2 ± 14.1	177.1 ± 31.5
Proline	185.9 ± 14.4	137.4 ± 35.6	158.0 ± 12.9	45.2 ± 27.4	314.9 ± 11.7	253.1 ± 47.3
Total	1,653.4 ± 238.7	1,198.6 ± 276.2	1,709.1 ± 310.5	1,187.2 ± 542.0	3,295.6 ± 557.4	2,236.7 ± 629.6

a: Expressed as mean ± S.D. of triplicate. b: A~F: different brands of chicken essences. c: Not detectable.

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Table 6. Constituted amino acids of peptides contents (mg/100 g)[a] in beef, clam and eel essences

	Beef	Hard clam	Freshwater clam	Eel
Phosphoserine	–[b]	2.2 ± 0.8	3.3 ± 0.3	–
Taurine	–	6.1 ± 27.9	4.7 ± 1.6	6.7 ± 11.1
Aspartic acid	314.8 ± 48.1	42.0 ± 3.2	16.3 ± 3.2	167.6 ± 71.4
Threonine	104.9 ± 12.7	14.3 ± 1.8	6.6 ± 1.8	63.9 ± 28.5
Serine	110.3 ± 18.5	15.4 ± 2.6	7.5 ± 1.2	78.9 ± 34.3
Glutamic acid	353.4 ± 270.6	26.4 ± 25.4	13.2 ± 8.2	190.0 ± 184.6
Glycine	311.8 ± 33.8	37.9 ± 4.8	14.8 ± 2.0	346.1 ± 141.8
Alanine	202.8 ± 25.9	33.9 ± 11.6	7.5 ± 2.4	173.0 ± 81.1
Valine	88.2 ± 17.5	7.1 ± 3.1	4.6 ± 3.1	45.2 ± 19.8
Methionine	41.4 ± 9.5	4.0 ± 6.4	0.5 ± 1.4	34.4 ± 21.8
Isoleucine	74.9 ± 34.2	9.6 ± 7.6	4.1 ± 1.4	38.5 ± 7.7
Leucine	116.9 ± 33.3	15.5 ± 5.8	6.7 ± 1.6	71.5 ± 42.0
Tyrosine	49.8 ± 10.6	9.9 ± 5.9	3.4 ± 2.0	25.8 ± 15.1
Phenylalanine	48.6 ± 13.6	8.8 ± 3.2	5.7 ± 0.8	31.6 ± 25.1
β-Alanine	24.6 ± 13.6	0.6 ± 1.2	7.6 ± 2.8	3.6 ± 4.9
Ornithine	4.2 ± 3.6	7.4 ± 6.0	33.2 ± 9.0	1.2 ± 1.8
Lysine	235.8 ± 28.5	29.6 ± 29.6	11.2 ± 3.4	94.8 ± 49.1
Histidine	105.7 ± 12.4	6.7 ± 3.9	2.9 ± 0.9	41.5 ± 20.1
Arginine	175.7 ± 16.9	32.7 ± 17.3	10.6 ± 7.6	108.8 ± 35.3
Proline	211.2 ± 16.4	11.2 ± 7.8	5.4 ± 9.4	175.8 ± 72.1
Total	2,567.0 ± 462.6	321.0 ± 25.2	222.8 ± 32.6	1,687.3 ± 861.2

a: Expressed as mean ± S.D. of triplicate. b: Not detectable.

peptides present in the product. Freshwater clam essence contained the lowest amount of peptides among all products. The dominant constituent amino acid in freshwater clam essence was ornithine, which accounted for 15% of the total peptides. As compared to freshwater clam essence, hard clam essence contained a lower level of ornithine in the peptide profile. Among the ten meat essences, hard clam essence was the only product in which the total peptide level was lower than its FAA.

The contributions of lower-molecular-weight peptides to bitterness, sweetness and umami taste have been reported[8,9]. With the inhibition activity of angiotensin I-converting enzyme, several specific peptides play an important role in antihypertensive activity[35,36], which may link with the fact that chicken essence can markedly suppress the elevation of blood pressure, cardiovascular hypertrophy and renal damage in hypertension rate[6,7]. In addition to carnosine and anserine, several peptides derived from protein hydrolyzates also possessed antioxidative activity[37,38]. With these findings in consideration, meat essences with high levels of small peptides may be benefitial to the consumers. However, the separation and identification of active peptides still need further investigation.

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市售雞精與其他肉精之一般成分、游離胺基酸與胜肽含量

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摘　要

十種市售肉精包括雞精、牛肉精、貝精與鰻精之水分含量介於91.1與97.5%之間，蛋白質0.6～7.8%及灰分0.3～1.7%，蜆精與文蛤精則另含高量肝醣。六種雞精游離胺基酸總量在150至725 mg/100 g之間，其中牛磺酸為主要成分，約佔總量之16~30%，雖然牛肉精之游離胺基酸量在所有產品中最高，其牛磺酸卻僅佔2%，蜆精之牛磺酸亦低，但富含鳥胺酸。甲肌肽在雞精中之含量為36～437 mg/100 g，高於肌肽之8～162 mg/100 g，而前者在牛肉精中則遠低於後者，此兩種雙胜肽在貝精中未被檢出，而鰻精亦僅含微量肌肽，另一雙胜肽之異甲肌肽則僅在一雞精中被發現。低分子量胜肽類之組成胺基酸總量在雞精中為1,187至3,296 mg/100 g，較各產品所含之總游離胺基酸高，兩種貝精所含胜肽類量遠低於其他產品，而文蛤精且為胜肽量低於游離胺基酸之唯一產品。

關鍵詞：雞精，肉精，一般成分，游離胺基酸，甲肌肽，肌肽，牛磺酸，胜肽類

EXHIBIT O

Bioactivities of Chicken Essence

Y.F. Li, R.R. He, B. Tsoi, and H. Kurihara

Abstract: The special flavor and health effects of chicken essence are being widely accepted by people. Scientific researches are revealing its truth as a tonic food in traditional health preservation. Chicken essence has been found to possess many bioactivities including relief of stress and fatigue, amelioration of anxiety, promotion of metabolisms and post-partum lactation, improvement on hyperglycemia and hypertension, enhancement of immune, and so on. These activities of chicken essence are suggested to be related with its active components, including proteins, dipeptides (such as carnosine and anserine), polypeptides, minerals, trace elements, and multiple amino acids, and so on. Underlying mechanisms responsible for the bioactivities of chicken essence are mainly related with anti-stress, anti-oxidant, and neural regulation effects. However, the mechanisms are complicated and may be mediated via the combined actions of many active components, more than the action of 1 or 2 components alone.

Keywords: anti-stress, carnosine, chicken essence

Introduction

In traditional medicine, chicken broth is regarded to have the effects of warming the body, countering body weakness and tonifying vigor, invigorating spleen and stomach, as well as strengthening tendons and bones. Chicken broth is suitable for alleviating the clinical symptoms of dystrophy, fear of cold, mental fatigue and lack of strength, menstrual irregularities, and postpartum agalactia. Originated from Ming dynasty of China, Wuji Baifeng pills have a history of more than a 100 y. These pills are made from black chickens and possess effects of nourishing blood and promoting fluid production, tranquillizing spirit, and enhancing intelligence. Combining the fact of rapid modern life rhythm and the important role of chicken broth in Chinese health preservation, chicken essence is conveniently accommodated into the fast paced modern life as an industrialized product and is prevalently consumed by Chinese in Southeast Asia. Chicken essence is mainly used to supply nutrition for patients, restore physical strength in postpartum women, improve physical quality of athletes, recover mental and physical fatigue, and enhance cognitive performance, particularly learning and memory. Besides, several lines of chicken essence products are enhanced with other traditional Chinese herbs to cater to the various needs of consumers. Because of its abundant physiological activities, chicken essence has been widely accepted by consumers, and a mature consumer market is gradually being developed. In an effort to provide useful information for consumers and further researches on chicken essence, this article summarizes the recent studies on the possible mechanisms underlying the bioactivities of chicken essence.

Active components of chicken essence

Compared with most other kinds of meat, chicken is rich in proteins, trace elements, carnosine, creatinine, and amino acids but has less fat. These ingredients can easily dissolve in and migrate to chicken soup by heating and boiling. However, due to the strictly controlled extraction processes, the ingredients proportion and their bioactivities in chicken essence are different from that in chicken broth. For example, Brand's Essence of Chicken (BEC), which has a history more than 170 y, is produced via a water extraction process from chicken meat for several hours under high-temperature, followed by centrifugation to remove fat and cholesterol, vacuum concentration to 3- to 4-fold, sterilization by high temperature and pressure before bottling. This kind of chicken essence is rich in protein, and low in sugar and fat, conveniently available and consumable, and easy for household storage. Previous studies confirmed that BEC not only contains indispensable amino acids including threonine, valine, methionine, isoleucine, leucine, phenylalanine, lysine, and tryptophan, but also contains a substantial proportion of free amino acids such as histidine and arginine (shown in Table 1) (Geissler and others 1996; Zain and Jamalulail 2003). Besides, many minerals, trace elements, vitamins, and some special nutrients such as anserine, carnosine, and taurine are also detected in BEC.

Effects of chicken essence on stress and fatigue

As lifestyle changes in modern society, physical tiredness is no longer the main reason responsible for fatigue. Nowadays, fatigue mainly derives from various kinds of stresses caused by environment and living habit alterations, physical and chemical factors, as well as psychological pressure (Maghout-Juratli and others 2010). Therefore, stress-related diseases have received considerable attention, and it is important to relieve stress for the goal of health maintenance. According to theory in traditional health preservation, chicken broth can relieve fatigues. Nagai and others (1996) evaluated the effect of chicken essence on mental fatigue in 20 healthy young students. Their results indicated that, after consuming 140 mL chicken essence per day for 7 d continuously, the error rates for the 2 mental workload tests, including mental arithmetic test and short-term memory test, were decreased significantly when compared to placebo control group. Meanwhile, they also evaluated the effects of chicken essence on the performance and emotional status of subjects by determining blood cortisol level, which is a stress hormone. The results suggested that basal cortisol

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levels of 2 groups were similar, while their cortisol levels increased significantly before mental workload tests, indicating their high stress status. After mental workload, the recovery of mean cortisol level of subjects who consumed chicken essence was significantly faster than those consuming placebo. Besides, they also found that subjects felt more active and less fatigued during the workload when they took chicken essence regularly. They concluded that chicken essence could enhance cortisol metabolism in blood and promote mental fatigue recovery.

It is well known that consuming tea or coffee could help focusing and consuming cocoa could relieve fatigue. Their mechanisms may be related with the activation of brain adenosine receptors by caffeine and theobromine. However, the anti-stress and anti-fatigue mechanisms of chicken essence are distinct from the previously mentioned drinks. It may relate to the activation of central histaminergic system (Lv and others 2010). It was shown in recent studies that compounds such as carnosine and anserine, which are important nutrients in chicken essence, are histamine dipeptides and antioxidants with multiple bioactivities. They are precursors in the synthesis of histamine, which can regulate central histamine and 5-hydroxytryptamine (5-HT) levels. This directly or indirectly decreased levels of stress-related substances such as cortisol during fatigue (Chen and others 2004; Yamano and others 2001), and regulated serotonin-dependent physiological activities, such as improving sleep quality, enhancing motivation, normalizing circadian rhythm, and so on. Besides, Lo and others (2005) also suggested that the anti-fatigue mechanism of chicken essence may be related with the regulation of plasma lactic acid and ammonia levels.

Effects of chicken essence on anxiety

Anxiety is a common mental disorder. Generally, it is believed that about 5% of world population suffer or experience light or moderate anxiety. Since anxiety is difficult to cure and most anti-anxiety drugs have various degrees of side effects, a combination treatment with psychotherapy and drugs as well as exercises is applied to relieve anxiety symptoms. Researchers suggested that nutritional interventions could be beneficial to both brain functions and symptoms associated with anxiety. Azhar and others (2001) investigated the effects of chicken essence combined with psychotherapy in 22 patients suffering anxiety. Results showed that chicken essence could enhance the cognitive abilities of patients, suggesting the beneficial effects of chicken essence on anxiety.

The anti-anxiety mechanism of chicken essence could be associated with regulations of histamine, 5-HT or other neurotransmitter pathways. As early as 1984, Watanabe and others (1984) successfully studied the distribution of histaminergic neuron system in the central nervous system of rats with histidine decarboxylase as a marker. Further studies showed that histaminergic neuron system was relevant to many brain functions, such as auto kinetic movement, sleep cycle, waking state, appetite control, learning, memory, and emotion, by employing pharmacological reagents, knock-out rats and positron emission tomography. Watanabe and Yanai (2001) reported that histaminergic system plays an important role in the regulation of central hyperactivity induced by food-deprived activity stress. However, we found that chicken essence had protective effects and suggested that these effects were partially correlated to carnosine, which is a histamine precursor compound abundant in chicken essence and plays important roles on central histaminergic system (Kurihara and others 2001; Lv and others. 2010). Moreover, the activity of 5-HT, another anxiety-related important neurotransmitter, was also indicated to be regulated by chicken essence. Xu and Sim (1997) found that chicken essence markedly increased the level of 5-hydroxyindoleacetic acid (5-HIAA), a main metabolite of 5-HT, in cerebrospinal fluid. This result reflected the influence of chicken essence on cerebral 5-HT metabolism. However, whether amino acids in chicken essence such as taurine, aspartate, glutamate, and glycine directly participate in regulation of 5-HIAA level still remains unknown.

Table 1– Active components of Brand's Essence of Chicken (BEC) (adapted from Geissler and others 1996; Zain and Jamalulail 2003).

Ingredient	Amount
protein (peptide)	83.0 mg/mL
Free amino acid	3.1 mg/mL
L-anserine	2.3 mg/mL
L-carnosine	0.8 mg/mL
Taurine	0.7 mg/mL
Hexos	0.8 mg/mL
Phosphatidyl collin	0.4 mg/mL
Minerals	
Calcium	26 μg/mL
Iron	1 μg/mL
Zinc	2 μg/mL
Magnesium	32 μg/mL
Potassium	1740 μg/mL
Natrium	550 μg/mL
Chlorine	1340 μg/mL
Phosphorus	480 μg/mL
Sulfur	500 μg/mL
Copper	2 μg/mL
Manganese	5 μg/mL
Selenium	0.05 μg/mL
Vitamins	
Vitamin B2	1.0 mg/kg
Vitamin B6	0.37 mg/kg
Vitamin B12	0.002 mg/kg
Niacin	6.4 mg/kg
Falacin	0.15 mg/kg
Vitamin C	15 mg/kg

Effects of chicken essence on glucose and lipid metabolisms

Fatigue not only affects central neurotransmitters and subsequently results in mental lassitude, but also induces bad appetite, which may be related with energy metabolism disorder. To maintain normal physiological functions, it is necessary to obtain energy from dietary glucoses and lipids. Stress was found to affect energy metabolism, limit energy uptakes of tissues and organs, disturb physiological functions, and lead to fatigue (Roberts and Sindhu 2009). Taketani and others (1998) reported that 2 dynamic hormones, cortisol and dehydroepiandrosterone (DHEA), had dual-direction regulation on glucose and lipid metabolisms, and at the same time controlled the occurrence of fatigue. Previously, our laboratory observed that blood cortisol level was significantly increased in 55 healthy adults who worked all night (Kurihara and others 2003), while Nagai and others (1996) confirmed that chicken essence reduced cortisol level after stress load. Recently, we found that restraint stress significantly reduced blood insulin level and decreased hepatic glycogen synthesis in mice. In addition, we also found that restraint stress could increase the levels of blood ketone bodies, and decrease the clearance of blood glucose in mice loaded with sucrose (Kurihara and others 2006). Restraint stress was also demonstrated to inhibit the activity of lipoprotein lipase in fat tissue, and reduce the clearance of blood neutral fat in mice loaded with Intralipid (He and others 2009a). However, chicken essence was indicated to increase lipoprotein

lipase activity in fat tissues, and improve blood lipid metabolism in stressed mice. Apart from lipid metabolism, chicken essence was also observed to increase insulin level, promote hepatic glycogen synthesis and blood glucose utilization in stressed mice (Kurihara and others 2006). These indicated that chicken essence could protect stress-mediated dysfunction in glucose and lipid metabolism, suggesting its benefits against fatigue.

Effects of chicken essence on energy metabolism

In the study of the effects of chicken essence on resting metabolism rate, Ikeda and others (2001) found that resting energy expenditure (REE) values after consuming chicken essence tablets were significantly higher than those consumed skim milk protein tablets (control trial). The increased thermogenic effects were continued at least for 1 h and then gradually decreased towards the baseline. The REE values during control treatment did not show such an augmented response. Geissler and others (1989) tested the thermic response after chicken essence consumption in 2 sets of 20 young healthy male and female subjects. Results showed that metabolic rate was significantly increased by 10% to 12% after consuming 70 mL of chicken essence for 2 h. They also confirmed that the effects of chicken essence on metabolism rate were greater than that of chicken broth. In another experiment, they found that consuming 70 mL of chicken essence with 30 to 40 kcal calories and a mixture of foods with 250 to 300 kcal had equal effects on metabolism. The response was greater than expected from the total energy and protein content of chicken essence, which may be due to the particular amino acid composition, other specific components and/or a cephalic response to taste. Tsi and others (2003) found that the combined thermogenic effect of capsaicin, green tea extract, and chicken essence could translate to a positive clinical effect by reducing approximately 460 g of body fat, following 2 wk of supplementation.

Effect of chicken essence on hyperglycemia

According to traditional health preserving experiences, people believe that frequent consumption of chicken essence by diabetics is beneficial to their health. Yamano and others (2001) observed the effects of chicken essence and its active component carnosine on 2-deoxy-D-glucose (2DG)-induced hyperglycemia by intracranial injection in rats. They found that the elevated blood glucose level induced by 2DG was notably decreased after consuming 5% chicken essence for 7 consecutive days. They also confirmed that carnosine, administrated intraperitoneally, intracerebroventricularly, or intragastrically, was effective to 2DG-induced hyperglycemia in rats. Moreover, they demonstrated that the anti-hyperglycemic effect of carnosine was related to the elevation of blood insulin level and reduction of glucagon level. In this experiment, Yamano and others (2001) also noticed that intravenous injection of certain doses of carnosine could inhibit the activity of sympathetic efferent nerves, which innervate the adrenal gland and liver, and reinforce the activity of parasympathetic efferent nerves, which innervate the pancreas. At the same time, certain doses of histamine were also found inhibiting 2DG-induced hyperglycemia in rat. Their experimental results showed that the inhibition of 2DG-induced hyperglycemia in rat by intracerebroventricular injection of histamine or carnosine could be counteracted by thioperamide, which is histamine H_3 receptor antagonist. The researchers suggested that carnosine, which act as precursor of histamine and is abundant in mammalian skeletal muscles, may act on the autonomic nervous system via H_3 receptors and thereby reducing blood glucose levels. However, further confirmations on the mechanism of action and conduction pathway are still needed.

In addition, our laboratory has proven that carnosine can accelerate blood glucose elimination rate, improve glycogen synthase gene expression, and enhance the ability of glycogen synthesis in restraint stressed mice (Yang and others 2010). The mechanism can be related to the improvement of glucocorticoid level and the activation of glycogen synthase kinase by carnosine, which then lead to dephosphorylation of glycogen synthase. It is well known that the sympathetic-adrenal medullary system will be activated as a response to stress. Large amounts of catecholamine and excitatory amino acids will then be released, which in turn hyper-activate the hypothalamic-pituitary-adrenal axis (HPA axis) and elevate blood corticosteroid level (Tsigos and Chrousos 2002). The augmentation of glucocorticoid release not only promotes glycogen degradation and gluconeogenesis, but also inhibits transportation and utilization of glucose in peripheral tissues. Blood glucose is then promptly increased under this condition. Administration of carnosine can lower blood glucose level in restraint stressed mice. It can also improve stress-induced decrease of blood glucose utilization. At the same time, carnosine improved stress-induced energy insufficiency as seen by significantly dropped plasma corticosterone level, promoted glycogen synthesis, lowered glucocorticoid contents, and increased glucose storage in restraint stressed mice. Glycogen synthase is an important rate-limiting enzyme for the synthesis of liver and muscle glycogen. Glycogen synthase-2 is mainly found in liver and regulates the synthesis of liver glycogen. Through the regulation of cAMP and Ca^{2+}, glucocorticoid causes phosphorylation of glycogen synthase by activating glycogen synthase kinase, which lowers glycogen synthase activity (Bollen and others 1998; Exton 1987).

Effects of chicken essence on immune function

In the study of effects of chicken essence on immunity, Man and others (2005) showed that chicken essence could increase the activity of serum immunoglobulin in normal and stressed animals. Candlish (1998) demonstrated that chicken essence has a stimulatory effect on human circulating neutrophils. They concluded that these effects may be due to the alcohol soluble micromolecules, of which carnosine is present in, and alcohol precipitated macromolecules. Their study also indicated that the macrophage-like cell line U937 could be activated by chicken essence *in vitro*. Recently, our laboratory investigated the effects of chicken essence on immune function in mice loaded with restraint stress and the results showed that chicken essence inhibited spleen lymphocytes apoptosis to maintain immune cell number and protect immune functions (Li and others 2010). We further found that carnosine, which showed similar immune effects with chicken essence on function, was the active substance in chicken essence (unpublished data).

Influenza is an acute infectious disease caused by RNA viruses of the Orthomyxoviridae family (the influenza viruses). Influenza virus infections often occur in the elderly population and individuals presenting fatigue or stress, who have weakened immunity (Dushoff and others 2006). Previous studies demonstrated that fatigue could induce oxidative stress in immune cells and result in immune dysfunction (Piche and others 2008). Fatigue was also demonstrated to affect nasal ciliary motility and increase the probability of microorganism infections. Besides, stress has been shown to affect the number and cytotoxicity of nature killer (NK) cells and induce significant changes in immune response of host towards viral or bacterial pathogens (He and others 2011). Recently, we

have conducted a study to investigate anti-influenza virus effect of chicken essence in mice loaded with restraint stress. Results showed that chicken essence could prevent influenza infection by modulating immune functions, although it could not directly treat influenza as a drug (Wang and others 2011).

Effects of chicken essence on lactation

According to the theory of traditional Chinese medicine, the reasons for postpartum hypogalactia include "Xu" (deficiency) and "Shi" (excess). "Xu" means blood deficiency, spleen, and stomach dysfunction, fluid exhaustion, loss of blood, and so on. It may cause lactation failure and result in postpartum hypogalactia. "Shi" means stagnations of meridian and blood. It may cause galactostasis and result in postpartum hypogalactia. Therefore, puerperal with "Xu" should be treated by tonification, while those with "Shi" should be treated by dredging. In reality, hypogalactia in puerperals are mostly caused by "Xu" and tonification is needed during the first few days of postpartum. Li and Li (1997) showed that chicken essence consumption could promote post-partum lactation and increase the production of milk. Besides, Chao and others (2004) examined the effects of chicken essence on lactation in 30 lactating women and found that chicken essence significantly elevated the contents of lactoferrin and epidermal growth factor (EFG), which are important for infants growth and contribute to the improvement of their immunity.

Effects of chicken essence on anemia

Chicken essence is widely used as a traditional remedy for several ailments, including anemia. A series of experiments was carried out in anemic rats by Geissler and others (1996) and results demonstrated that chicken essence could improve hemoglobin levels and indicated that the effects were mediated by increased appetite and enhanced availability of dietary iron. On one hand, chicken essence could increase appetite through stimulating the secretion of gastric juice. The increased appetite resulted in more consumption and thus the absorption of more nutrients from diet, which would be required for the synthesis of hemoglobin. On the other hand, many nutrients such as vitamins and amino acids can stimulate the absorption and usage of iron from food. It is generally recognized that iron obtained from food is non-heme iron, which cannot be utilized until it is dissolved and reduced. Earlier researches demonstrated that various factors, such as vitamin C and citric acid, could affect the dissolution and reduction processes of non-heme iron obtain from food. Carnosine and anserine, which are abundant in chicken essence, were also indicated to affect the absorption of iron through their anti-oxidative and metal chelating effects. However, the role of chicken essence in prevention of anemia needs to be further elucidated.

Effects of chicken essence on hypertension

In recent years, studies have proven that the angiotensin converting enzyme (ACE) inhibitory peptide obtained from protease hydrolyzed chicken protein is safe, easily absorbed into the body and inhibits the activity of ACE in human body. Zhang and others (2007) proved that among the chicken-originated bioactive peptides, ACE inhibitory peptide was one of the antihypertensive peptides, which has strong application prospect as a functional component in food. Saiga and others (2003) extracted 3 peptides from chicken extract which possess hypotensive activity by inhibiting ACE. These peptides were found to have a common sequence, Gly-X-X-Gly-X-X-Gly-X-X, among which Gly-Phe-Hyp-Gly-Thr-Hyp-Gly-Leu-Hyp-Gly-Phe showed the strongest ACE inhibitory activity. Matsumura and others (2001) used deoxycorticosterone acetate salt (DOCA-salt) induced hypertension model after unilateral nephrectomy in Sprague–Dawley rats to investigate the effect of chicken essence towards hypertension. It turned out that administration of freeze-dried chicken essence powder, which is equivalent to 0.175 to 0.35 mL chicken essence, once a day for 5 wk could notably lower DOCA-salt induced hypertension in rats. Moreover, chicken essence improved cardiac histology alteration caused by DOCA-salt. Physiological examination revealed that chicken essence can alleviate the thickened aortic wall and increased aortic wall area induced by DOCA-salt. Chicken essence could also alleviate renal damages induced by DOCA-salt, like glomerular fibrous necrosis, renal artery thickening, and tubular expansion. Matsumura and others also noticed that chicken essence could inhibit the increase of urine protein induced by DOCA-salt. Ririe and others (2000) proved that carnosine is beneficial to vascular endothelium-independent vasodilation of aorta in rats. It is believed that the generation of oxygen radicals is increased in hypertension animals, while oxidative stress is closely related to hypertension. Thus, we can infer that the anti-oxidant and free radical clearing carnosine could alleviate hypertension and organic pathologic histology alterations. Slim (2001) also confirmed that chicken essence could relieve overload myocardial hypertrophy, hypertension, atherosclerosis, and so on. When comparing the anti-myocardial hypertrophic effects of chicken essence with extracts from pork, the cardiovascular improving effects of chicken essence is well worth attention. Chicken meat extract also showed stimulating activity on the production of NO by macrophage. This may be related to its effect on improving cardiovascular functions. Taken together, from a nutritional point of view, long-term consumption of chicken essence may have certain preventive and curing effects on hypertension.

Other bioactivities

Investigation revealed that stress-induced physical and mental fatigue is related to the increment of lipid peroxidation (LPO) (Kennedy and others 2005). For example, LPO amount in urine of tired people is in inverse proportion with flicker fusion threshold (the index that evaluates brain functions) and in direct proportion to lactic acid concentration in blood. We also discovered that working overnight caused brain fatigue and lowered calculation ability, and was associated with increased blood LPO. Ou and others (2001) suggested that the Eastern concept of yin and yang constraints parallels the Western concept of the balance of peroxidation and anti-oxidation. Abidin and others (2004) also confirmed this phenomenon. When people are continuously depressed, certain degree of brain ischemia and production of reactive oxygen species may occur. When peroxidized lipids, product of oxidative damage, are accumulated in cerebral cortex, physical and mental faculties may be affected adversely. In addition, we found that physical and mental pressure in mice was induced by restraint stress. At the same time, the oxygen radical absorbance capacity was significantly decreased (He and others 2009b; Kurihara and others 2009). It can be seen that stress load not only causes fatigue but also induces endogenous oxidative stress. In the past few years, studies found that small molecule compounds like carnosine and anserine could enhance the anti-oxidative capacity of body, tissues and cells. Kurihara and others (2009) indicated that carnosine could improve the internal oxidative stress induced by fatigue. Zhou and others (2006) indicated that the hydrolysates, obtained

from hydrolysis of chicken protein by alkaline protease, possess notable clearing activity of hydroxyl and superoxide free radicals. Lai and Guo (1999) indicated that carnosine extracted from chicken meat has LPO inhibiting effects. The anti-oxidant activity of carnosine is related with the reductive and metal chelating characteristic (Kohen and others 1988). Carnosine can form non-reactive adducts with various free radicals such as lipid peroxyl radicals and hydroxyl radicals, thus demonstrating a strong free radicals capture ability (Salim-Hanna and others 1991). Carnosine also possesses non-enzymatic glycating activity (Hipkiss and Chana 1998). As carnosine can competitively react with aldehydes and ketones, it can lower the content of active carbonyls, inhibit protein cross-linking and relieve the damaging effects of free radical to biomacromelecules. We have confirmed that, although *in vitro* anti-oxidant activity of carnosine is not obvious, oral administration of carnosine shows apparent anti-oxidant and anti-stress effects (unpublished data). However, the mechanisms of action still need further investigation in the future.

Besides, Wu and others (2011) had reported the effect of chicken essence on the adaptation of circadian clocks to experimental jet lag in rats. They found that chicken essence markedly facilitated the re-entrainment of all examined clock genes (Bmal1, Cry1, Per1, and Per2) in the pineal gland, reduced from 7 d to only 3 to 5 d. In the liver clock, chicken essence was also indicated to shorten the resetting of Bmal1 and Per2 by nearly 2 d. However, the resetting rate of locomotor activity rhythm was not affected by chicken essence, suggesting that it might be unable to affect behavioral rhythm.

Moreover, chicken essence is a traditional functional food, which is rich in diversified nutrients, flavor, and it can enhance appetite and help digestion. Its function is being paid attention in clinical nutrition. The clinical results of Wang (2006) confirmed that chicken essence used in the process of enteral nutrition therapy could improve patients' health. Another research indicated that chicken essence can effectively protect intestinal functions and reduce the occurrence of complications during enteral nutrition therapy (Wang 2007). In addition, chicken essence also has other physiologic functions such as improving physical fitness in athletes and enhancing responsiveness of the brain.

Table 2–Summary of bioactivities and mechanisms of chicken essence.

Bioactivities	Mechanisms	Possible functional substances and their effective dosages	References
Anti-stress Anti-fatigue	Activate central histaminergic system; Regulate plasma lactic acid and ammonia levels	Carnosine: 50 and 200 mg/kg (orally administrated to mice) Anserine: 322 mg/kg (orally administrated to mice)	Nagai and others (1996); Lo and others (2005); Lv and others (2010)
Anti-anxiety	Regulate histamine, 5-HT or other neurotransmitter levels	Carnosine: 50 and 200 mg/kg (orally administrated to mice)	Xu and Sim (1997); Azhar and others (2001); Kurihara and others (2001); Lv and others (2010)
Promoting metabolisms	Increase insulin level, promote hepatic glycogen synthesis and blood glucose utilization; Increase lipoprotein lipase activity and improve blood lipid metabolism	High molecular peptides fraction: 200 mg/kg (orally administrated to mice); Low molecular peptides fraction (containing carnosine and anserine, and so on): 200 mg/kg (orally administrated to mice)	Geissler and others (1989); Ikeda and others (2001); Tsi and others (2003); Kurihara and others (2006)
Promoting postpartum lactation	Increase the production of milk; Elevate the contents of lactoferrin and epidermal growth factor (EFG)	Unclear (proteins, carnosine, anserine and minerals may be the possible contributors)	Li and Li (1997); Chao and others (2004)
Anti-hyperglycemia	Reduce blood glucose level through the regulation of autonomic nerves by histamine H_3 receptor; Elevate blood glucose elimination rate, activate glycogen synthase kinase through improving glucocorticoid level	Carnosine: 0.005 to 5 nmol (injected into peritoneal of rats); 0.05 to 0.5 nmol (injected into right lateral cerebral ventricle of rats); 150 to 300 mg/kg (orally administrated to mice)	Yamano and others (2001); Yang and others (2010)
Anti-hypertension	Inhibit the activity of ACE by peptides; Alleviate the thickening of aortic wall and reducing aortic wall area through anti-oxidant and free radical clearing activities of carnosine	ACE inhibitory peptides: such as Gly-Phe-Hyp-Gly-Thr-Hyp-Gly-Leu-Hyp-Gly-Phe (inhibitory activity against ACE was 42 μM); Carnosine: 33.3 mg/kg (orally administrated to rats)	Ririe and others (2000); Matsumura and others (2001); Slim (2001); Saiga and others (2003)
Enhancing immunity	Increase the activity of serum immunoglobulin; Stimulate circulating neutrophils; Inhibit immunocyte apoptosis	70% alcohol soluble micromolecules, possibly carnosine and anserine: stimulatory effect on human circulating neutrophils at 10 mM *in vitro*; 70% alcohol precipitated macromolecules, possibly containing protein: dosage is unclear	Candlish (1998); Man and others (2005); Li and others (2010)
Improving anemia	Increase appetite and enhance availability of food iron to improve hemoglobin level	Unclear (may be the combined actions of vitamins, amino acids, carnosine, anserine, and minerals)	Geissler and others (1996)

The mechanisms of the bioactivities of chicken essence are complicated and may be mediated via the combined actions of many functional substances, more than the action of 1 or 2 substances alone.

Conclusions

Chicken essence, derived from a strictly controlled proprietary process of extraction and concentration, contains diverse nutrients and possesses many significant bioactivities with little adverse effects. It is recommended that people, who are under high pressure, hypertensive, or diabetic, can often consume chicken essence to relieve stress and fatigue, enhance immunity, ameliorate anxiety, improve learning and memory, and improve glucose or lipid metabolism. The bioactivities and the mechanisms of chicken essence are summarized in Table 2. What is worth mentioning is that the mechanisms are complicated and may be mediated via the combined actions of many functional substances, more than the action of 1 or 2 substances alone. The industrialization and diversification of chicken essence can be accomplished using the combination of traditional chicken essence preparation techniques and modern biotechnology, so that chicken essence may exert its full beneficial effects on elevating human health and life quality.

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EXHIBIT P

UNITED STAT[illegible] DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR		ATTORNEY DOCKET NO.
09/757,78[illegible]	01/[illegible]/01	HARRIS	R	SD-08457-GPE

HM22/0815

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EXAMINER	
HENLEY III, R	
ART UNIT	**PAPER NUMBER**
1614	5

DATE MAILED: 08/15/01

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner of Patents and Trademarks

PTO-90C (Rev. 2/95)
*U.S GPO 2000-473-000/44602

1- File Copy

Office Action Summary	Application No. 09/757,782	Applicant(s) Roger Harris, et al.
	Examiner Ray Henley	Art Unit 1614

-- *The MAILING DATE of this communication appears on the cover sheet with the correspondence address* --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136 (a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
- Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b)

Status

1) ☒ Responsive to communication(s) filed on *Aug 3, 2001*

2a) ☒ This action is **FINAL**. 2b) ☐ This action is non-final.

3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11; 453 O.G. 213.

Disposition of Claims

4) ☒ Claim(s) *1-47* is/are pending in the applica

4a) Of the above, claim(s) ______ is/are withdrawn from considera

5) ☒ Claim(s) *34-40 and 43* is/are allowed.

6) ☒ Claim(s) *1-26, 29-33, 41, 42, and 44-47* is/are rejected.

7) ☒ Claim(s) *27 and 28* is/are objected to.

8) ☐ Claims ______ are subject to restriction and/or election requirem

Application Papers

9) ☐ The specification is objected to by the Examiner.

10) ☐ The drawing(s) filed on ______ is/are objected to by the Examiner.

11) ☐ The proposed drawing correction filed on ______ is: a) ☐ approved b) ☐ disapproved

12) ☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. § 119

13) ☐ Acknowledgement is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d)

a) ☐ All b) ☐ Some* c) ☐ None of.

1. ☐ Certified copies of the priority documents have been received.
2. ☐ Certified copies of the priority documents have been received in Application No. ______ .
3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

*See the attached detailed Office action for a list of the certified copies not received.

14) ☐ Acknowledgement is made of a claim for domestic priority under 35 U.S.C. § 119(e)

Attachment(s)

15) ☒ Notice of References Cited (PTO-892)

16) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)

17) ☐ Information Disclosure Statement(s) (PTO-1449) Paper No(s) ______

18) ☐ Interview Summary (PTO-413) Paper No(s). ______

19) ☐ Notice of Informal Patent Application (PTO-152)

20) ☐ Other

CLAIMS 1-47 ARE PRESENTED FOR EXAMINATION

Applicants' amendment filed August 3, 2001 has been received and entered into the application. Accordingly, the specification at page 1 has been amended and claims 13-47 have been added.

It is noted at page 6 of the amendment that the cancellation of claims 1-12 was intended, however, under the "amendment" section at pages 2-5, there fails to be any specific direction for the cancellation of claims 1-12. Accordingly, claims 1-12 remain pending.

Double Patenting

Statutory

Claims 1-12 remain rejected under 35 U.S.C. 101 as claiming the same invention as that of claims 1-12 of prior U.S. Patent No. 6,172,098, already of record, for the reasons of record as set forth in the previous Office action dated April 3, 2001. Insofar as claims 1-12 remain pending, the rejection is deemed to remain proper.

Obviousness-type

I Claims 13-21 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 3-4 of U.S. Patent No. 6,172,098 (Harris et al.), already of record. Although the conflicting claims are not identical, they are not patentably distinct from each other because the selection of an appropriate dosage form and dosage amounts

would have been matters well within the purview of the skilled artisan. Also, the patented claims provide for a peptide source and the selection of any particular peptide would have been a matter well within the purview of the artisan. Finally, the composition of present claims recite comprising and thus are open for the inclusion of ingredients such as those of the patented claims.

II Claims 22-26, 29-33 and 44-47 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-2 of U.S. Patent No. 6,172,098 (Harris et al), already of record. Although the conflicting claims are not identical, they are not patentably distinct from each other because the selection of an appropriate dosage form and dosage amounts would have been matters well within the purview of the skilled artisan. Also, the patented claims provide for a carbohydrate in general and the selection of any particular carbohydrate would have been a matter well within the purview of the artisan. Finally, the composition of present claims recite comprising and thus are open for the inclusion of ingredients such as those of the patented claims.

Claim Rejection - 35 USC § 112, Second Paragraph

Claims 19, 20, 31, 32, 41 and 42 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

The claims are considered indefinite because the limitation "per kilogram of body weight" depends upon a feature not appearing in the claim and not related to any physical and/or structural

feature of the composition. A proper limitation as to the amount/proportion of the ingredients present in a given composition would relate the amount of the ingredient to either the volume or weight of the composition. In claims directed to a method of treatment which involves the administration of a composition to a subject, it is proper to relate the amount of an ingredient in a composition to the weight of the subject because the presence of the subject is a positive limitation of the claimed method. This, however, is not the present situation.

Claim Rejection - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless --
>
> (e) the invention was described in a patent granted on an application for patent by another filed in the United States before the invention thereof by the applicant for patent, or on an international application by another who has fulfilled the requirements of paragraphs (1), (2), and (4) of section 371© of this title before the invention thereof by the applicant for patent.

Claims 44 and 45 are rejected under 35 U.S.C. 102(e) as being anticipated by De Lacharriere et al. (U.S. Patent No. 5,976,559) who teach pharmaceutical compositions comprising beta-alanine to be old (see, for example, column 4, lines 6 and 51-57).

The functional language in the present claims has been considered, but fails to impart any physical or otherwise material property to the composition that is not found in the prior art compositions.

Claim Rejection - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

Claims 44 and 45 are rejected under 35 U.S.C. 103(a) as being unpatentable over De Lacharriere et al. (U.S. Patent No. 5,976,559), as above.

The difference between the above and applicants' claimed subject matter lies in that the prior art does not highlight the various forms of beta alanine as presently claimed.

However, to the skilled artisan, the claimed subject matter would have been obvious because it was well known that pharmaceutically active agents could also be successfully employed in common derivative forms such as their esters or amides.

Claim Objection

Claims 27 and 28 are objected to as depending from a rejected base claim, but are otherwise in condition for allowance.

Allowable Subject Matter

Claims 34-40 and 43 are deemed to be in condition for allowance.

Applicants' amendment necessitated the new grounds of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a). Applicants are reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Ray Henley whose telephone number is (703) 308-4652.

RAYMOND HENLEY, III
PRIMARY EXAMINER
GROUP 1600

Henley; rjh
August 13, 2001

Notice of References Cited	Applicant/Patent: Roger Harris, et al.	Application/Control No.: 09/757,782	
	Examiner: Ray Henley	Art Unit: 1614	Page 1 of 1

U.S. PATENT DOCUMENTS

		Document Number Country Code-Number-Kind Code	Date MM-YYYY[1]	Name	Classification[2]	
	A	5,976,559	11/1999	De Lacharriere et al.	424	401
	B					
	C					
	D					
	E					
	F					
	G					
	H					
	I					
	J					
	K					
	L					
	M					

FOREIGN PATENT DOCUMENTS

		Document Number Country Code-Number-Kind Code	Date MM-YYYY[1]	Country	Name	Classification[2]	
	N						
	O						
	P						
	Q						
	R						
	S						
	T						

NON-PATENT DOCUMENTS

		Include, as applicable: Author, Title, Date, Publisher, Edition or Volume, Pertinent Pages
	U	
	V	
	W	
	X	

* A copy of this reference is not being furnished with this Office action. See MPEP § 707.05(a).

[1] Dates in MM-YYYY format are publication dates. [2] Classifications may be U.S. or foreign.

8/13/04

U.S. Patent and Trademark Office
PTO-892 (Rev. 01-2001) Notice of References Cited Part of Paper No. 5

EXHIBIT Q

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: 0 449 787 A2

(12) EUROPEAN PATENT APPLICATION

(21) Application number: 91830107.8

(22) Date of filing: 19.03.91

(51) Int. Cl.[5]: A61K 37/02, // (A61K37/02, 31:205), (A61K37/02, 31:195), (A61K37/02, 31:205, 31:195)

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(84) Designated Contracting States: AT BE CH DE DK FR GB IT LI LU NL SE

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(54) **Pharmaceutical, dietetic or veterinary compositions with eumetabolic activity.**

(57) Pharmaceutical, dietetic or veterinary compositions containing carnosine or dipeptides related thereto having an activity on muscular metabolism, are described.

EP 0 449 787 A2

The present invention relates to pharmaceutical, dietetic or veterinary compositions containing carnosine or peptides related thereto as the active ingredient.

Carnosine is a physiological substance, which is present in muscles and in some nervous tissues of mammals and, particularly, of man, in concentrations ranging from 15 to 40 mmoles/ kg of tissue. Chemically, carnosine consists of a dipeptide, namely β-alanyl-L-histidine.

The physiological action of this peptide is well-known and it is expressed at intracellular level by buffering the exceeding protons, which come from the physiological processes involved in energy production.

In fact, after an increased muscular or cerebral activity, the so called "breathing-acidosis" occurs and, after an even stronger activity, glycogen degradation gives rise to lactic acid accumulation in tissues, and the consequent "metabolic acidosis" occurs.

In both cases uncontrolled proton release would cause an intracellular pH drop, so as to compromise the phosphocreatine-creatine phosphokinase system, which is involved in the regeneration of ADP to ATP; the failure of said system is probably one of the main causes of muscular fatigue.

Intracellular buffering agents act by shifting the point where phosphocreatine-creatine phosphokinase system blockage occurs, therefore lowering weakening sensation.

All dipeptides with pKa near physiological pH can act as intracellular buffering agents; in addition to carnosine, other dipeptides containing histidine imidazole ring can be used such as:

homocarnosine: α-aminobutyryl-L-histidine
anserine: β-alanyl-L-1-methyl-histidine
homoanserine: α-aminobutyryl-L-1-methyl-histidine
ophidine: β-alanyl-L-3-methyl-histidine.

It has now been found that administering these substances in the form of pharmaceutical, dietetic or veterinary compositions to human or animal organisms is capable of causing beneficial effects, which are unpredictable from what is known about the biochemistry and the metabolism of said physiological substances.

Examples of such beneficial effects include athletic performance improvement in persons subjected to prolonged efforts, improvement of muscular functional capacity in elderly or weakened subjects, in children, in dialysed patients or in those patients with a reduced hepatic functional capacity. Carnosine administration proved also to be advantageous in breeding, particularly in improving horse performances in equestrian sports. Therefore, according to the invention, oral pharmaceutical, dietetic or veterinary compositions containing a dipeptide selected from the group of carnosine, homocarnosine, anserine, homoanserine or ophidine or physiologically equivalent derivatives thereof such as salts, acetyl derivatives and the like are provided. Said compositions can further contain other active ingredients with complementary or anyway useful activities such as creatina, carnitine, acetylcarnitine, essential or non essential amino acids, sugars, mineral salts and vitamins. Carnosine is the preferred dipeptide, whose daily posology can vary within broad limits, becausa of its extremely low toxicity. In therapeutic or dietetic use, daily posology will generally range between 300 mg and 5 g a day. Other optional active ingredients, which are selected depending on the specific indications foreseen for tha compositions of the invention, will be present in such an amount to provida a suitable supply of said substances.

For instance creatine can be administered in doses from 0.5 to 10 g a day; carnitine in doses from 100 mg to 2 g a day, amino acids from 100 mg to 5 g a day; always referring to human therapeutic or dietetic use.

The compositions of the invention are prepared using conventional techniques and excipients, like the ones described in "Remington's Pharmaceutical Sciences Handbook", Mack Pub. Co., N.Y., U.S.A.

Examples of formulations include single-dose sachets containing powders or granulates which can optionally be effervescent and can be dissolved in water or other beverages before use; tablets; soft or hard capsules; syrups; sweets and the like; containing not only carnosine and optionally other active ingredients, but also appropriate excipients such as flavouring, sweatening, effervescence agents and all those additivas which are well-known to the man skilled in the art.

The following examples further illustrate the invention.

EXAMPLE 1

Composition, to be orally administerad from four times to twica a day as support to a strong muscular activity, containing carnosine, essential amino acids and sugars, in the following proportions:

Carnosine	1.0 g
Histidine	1.0 g
Carnitine	0.5 g
Creatine	2.0 g
Glucose	1.0 g
Flavouring agents	

EXAMPLE 2

Composition, to be administered to subjects with nutritional or liver metabolic deficiency or in case of muscular fatigue from three times to once a day, containing carnosine and essential amino acids in the following proportions:

Carnosine	0.35 g
Creatine	0.75 g
Histidine	0.35 g
Carnitine	0.175 g
Flavouring agents	

EXAMPLE 3

Carnosine	1.0 g
Potassium aspartate	1.0 g
Fructose or dextrose	1.0 g
Sodium hydrogencarbonate	1.5 g

EXAMPLE 4

Acetylcarnosine	0.8 g
Histidine	0.8 g
Carnitine	0.3 g
Potassium aspartate	1.0 g

Claims

1. Pharmaceutical, dietetic or veterinary compositions containing a dipeptide selected from the group of carnosine, homocarnosine, anserine, homoanserine or ophidine or physiologically equivalent derivatives thereof as active ingredient in admixture with a proper carrier.

2. Compositions according to claim 1, wherein the dipeptide is carnosine.

3. Compositions according to claim 1 or 2, characterized by containing one or more active ingredients selected from carnitine, creatine, essential or non essential amino acids, sugars, mineral salts, vitamins.

4. Compositions according to any one of claims 1-3 in the form of powders or granulates distributed in single-dose sachets.

5. The use of the dipeptides selected from the group of carnosine, homocarnosine, anserine, homoanserine, ophidine or physiologically equivalents thereof for the preparation of pharmaceutical, dietetic or veterinary compositions useful for treating muscular fatigue states and improving athletic performances in persons subjected to prolonged physical efforts.

EXHIBIT R

Eur. J. Biochem. 219, 43–47 (1994)

Biosynthesis of carnosine and related peptides by skeletal muscle cells in primary culture

Karl BAUER and Michael SCHULZ
Max-Planck-Institut für experimentelle Endokrinologie, Hannover, Germany

(Received August 6, 1993) – EJB 93 1201/1

Synthesis of carnosine (β-alanyl-L-histidine) and related dipeptides could be demonstrated in primary muscle cell cultures derived from embryonic chick pectoral muscle. After incubation with radiolabeled β-alanine or γ-aminobutyric acid, the radiolabeled dipeptides were isolated from the cell extracts and also in small amounts from the culture medium. The kinetics of dipeptide formation indicated that anserine (β-alanyl-1-methylhistidine) is not formed directly by these cells but as a secondary product via the methylation of carnosine. Coinciding with the morphological differentiation of the mononucleated myoblast to form multi-nucleated myotubes, a rapid increase in β-alanine uptake and also in dipeptide synthesis could be observed. These results demonstrate that carnosine and related peptides are not merely deposited in skeletal muscles but that they are actively synthesized by muscle cells in culture.

Carnosine was first isolated from Liebig's meat extract in 1900 [1] and was subsequently identified as β-alanyl-histidine [2, 3]. Since then, various ω-aminoacyl amino acids such as anserine (β-alanyl-1-methylhistidine) [4, 5], homocarnosine (γ-aminobutyryl-histidine) [6] and others have been isolated from excitable tissues [7, 8]. All these peptides are synthesized by carnosine synthetase [l-histidine:β-alanine ligase (AMP-forming)], an enzyme with broad substrate specificity [9, 10].

Although these peptides are present in relatively high concentrations and in some tissues even represent the major non-proteinaceous constituents [8], their physiological functions still remain enigmatic. Some suggestions concerning the role of carnosine in glycolysis, oxidative phosphorylation and muscle contraction [8] have not been verified. On the basis of biochemical studies it has been suggested that carnosine may be the principal neurotransmitter in the mammalian olfactory pathway [11]. Immunolocalization studies with antisera against carnosine and anserine support this hypothesis [12]. However, in non-olfactory areas of the central nervous system carnosine is not associated with neuronal cells, but with glial cells [12]. The latter findings are in agreement with our previous studies on the biosynthesis of carnosine and related peptides by the C6 glial cell line [13] and astroglia-rich primary cultures from rodent brain [14]. In skeletal muscle, the precise cellular localization of synthesis still remains unknown. Due to their high abundance, it is obvious that these peptides can only be stored in muscle tissue. Conceivably, however, these peptides could also be synthesized by motor neurons or Schwann cells and, after release, could subsequently be taken up and deposited in muscle cells. In this study, we demonstrate that chick muscle cells in primary culture actively synthesize and accumulate ω-aminoacyl amino acids.

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Enzyme. Carnosine synthetase [L-histidine: β-alanine ligase (AMP-forming)] (EC 6.3.2.11).

MATERIALS AND METHODS

Materials

Ham's F12 nutrient mixture and horse serum were obtained from GIBCO. Cells were grown in cell culture dishes (Nunc) or flasks (Falcon). β-[3(n)-^{3}H]Alanine, 120 Ci/mmol, and γ-[2,3(n)-^{3}H]aminobutyric acid, 40 Ci/mmol, were purchased from Du Pont de Nemours. Insulin, collagenase and hyaluronidase were from Sigma. Transferrin and bovine serum albumin were obtained from Boehringer Mannheim. Trypsin and Dowex 50 WX2 were from Serva and Aminex Q-15 S resin was obtained from Bio-Rad. Scintillator Hydroluma (J. T. Baker) was used for radioactivity measurements and all solvents were from Merck (Darmstadt). Embryo extract was prepared according to the procedure of Konigsberg [15].

Methods

Cell culture

Primary cultures of myoblasts were isolated from the pectoral muscle of 11-day-old chick embryos. The muscle tissue obtained from ten embryos was digested with 5 ml Puck's saline D2 solution (137 mM NaCl, 5.5 mM KCl, 0.22 mM KH_2PO_4, 0.168 mM Na_2HPO_4, 0.11 mM $CaCl_2$ and 5.5 mM glucose, pH 7.3) containing 0.25% collagenase and 0.025% trypsin. After 15 min at 37°C, the cell suspension was passed through a 125-μm nylon mesh into 10 ml cold growth medium (Ham's F12 medium containing 15% horse serum and 5% embryo extract). The cell suspension was centrifuged at 100×g for 5 min, the supernatant was aspirated and the cell pellet was dispersed in 20 ml fresh growth

medium. The cell suspension was transferred to a 250-ml culture flask to allow fibroblast attachment. After 30 min, unattached cells were recovered, counted and plated onto gelatin-coated culture dishes at a density of 8×10^5 cells/plate or 3×10^5 cells/plate, 90-mm or 60-mm diameter, respectively. The cells were grown at 37°C in a 5% CO_2-enriched atmosphere of humidified air. Half of the medium was replaced daily with fresh medium.

Tracer incorporation studies

The cells, grown for the time periods indicated, were incubated in appropriate fresh culture medium containing radiolabeled β-alanine or γ-aminobutyric acid. After incubation, the medium was aspirated and the cells were washed three times with 5 ml isotonic 10 mM potassium phosphate, 150 mM NaCl, pH 7.2 (NaCl/P_i). The washed cells were lysed by the addition of 1.5 ml methanolic acetic acid (2 M acetic acid in 60% methanol) and were scraped off the plates. After rinsing the plates twice with 1.5 ml of the same solution, the extract and the washings were combined. After extraction, the medium and the cell extracts were analysed separately by cation exchange chromatography either on an Aminex Q-15 S column [14] or on Dowex 50 WX2 resin [16] as described previously.

Protein determination

Protein was determined by the Lowry method as modified by Peterson [17] using bovine serum albumin as standard.

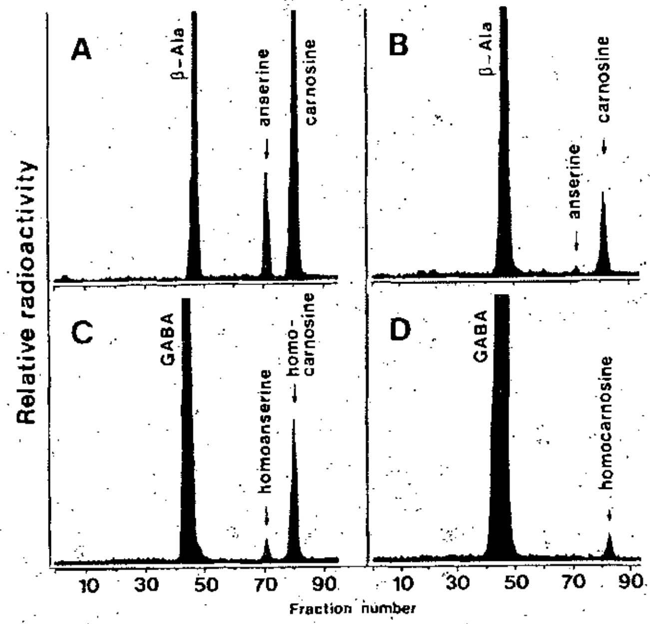

Fig. 1. The uptake and metabolism of radiolabeled β-alanine and γ-aminobutyric acid by muscle cells in primary culture. Muscle cells maintained in growth medium for 5 days were exposed to 2 µCi β-[^{3}H]alanine (A, B) or γ-[^{3}H]aminobutyric acid (C, D) dissolved in fresh medium. After a 48-h incubation, extracts prepared from cells (A, C) and medium (B, D) were resolved by cation exchange chromatography on an Aminex Q-15 S column. 25% of the column effluent was separated by stream splitting and continuously monitored for radioactivity by scintillation counting. GABA, γ-aminobutyric acid.

RESULTS

After 5 days in culture, the cells were incubated for 48 h with β-[^{3}H]alanine or γ-[^{3}H]aminobutyric acid. Extracts of the cells and the culture medium were prepared separately and subjected to ion-exchange chromatography as described in the Materials and Methods section. The column effluent was monitored continuously for radioactivity.

With β-[^{3}H]alanine as tracer, the fractionated cell extract displayed a very simple incorporation pattern. Besides β-alanine, we only detected radioactive material eluting exactly at the positions characteristic for anserine and carnosine, the main radiolabeled product (Fig. 1 A), as described previously [13]. The identity of the peptides was further evaluated by two-dimensional thin layer chromatography and by the identification of the constituent amino acids after acid hydrolysis, as described before [13]. Only small amounts of these peptides were found in the medium (Fig. 1B). In contrast to previous studies with C6 glioma cells [13] and astroglia-rich primary cultures [14], radiolabeled acidic products were neither detected in the cell extract nor in the culture medium.

When the cells were incubated with γ-[^{3}H]aminobutyric acid as radiolabeled precursor, degradation of the tracer to acidic products via transamination was also not observed. After incubation with γ-[^{3}H]aminobutyric acid, the amount of radioactivity recovered from the muscle cell extract was about the same as the amount recovered when β-[^{3}H]alanine was used, indicating that γ-[^{3}H]aminobutyric acid is also taken up very effectively by muscle cells. However, compared to carnosine and anserine, considerably less (29%) of the radiolabeled dipeptides homocarnosine and homoanserine could be isolated (Fig. 1C) and consequently only trace amounts of the γ-aminobutyric-acid-containing peptides could be recovered from the culture medium (Fig. 1D).

The time course of β-alanine uptake and dipeptide formation by muscle cells after 5 days in culture is shown in Fig. 2. After incubation with β-[^{3}H]alanine, radioactivity accumulates rapidly in the cells and the radioactivity in the medium declines. Carnosine is synthesized very rapidly, without an apparent lag phase, and accumulates in the cells. In contrast, the formation of anserine could only be detected after 8 h of incubation and only accounts for 7.5% of the radioactivity in the cells after incubation for 32 h. This result indicates that anserine is not formed directly but as a secondary product through the methylation of carnosine.

The uptake of β-alanine and the synthesis of carnosine by muscle cells as a function of time in culture are shown in Fig. 3. After plating and culturing for the time periods indicated, the cells were incubated for 16 h with β-[^{3}H]alanine. The uptake of β-alanine by the cells was followed by the decrease of radioactivity in the medium (Fig. 3). Carnosine synthesis (Fig. 3) was determined after separating β-alanine from carnosine by column chromatography. After the third and fourth day in culture, we observed a rapid increase in the rate of β-alanine uptake and also a dramatic increase in the amount of carnosine that could be isolated from the cell extract. This increase in dipeptide formation is not only the result of the increased uptake and thus availability of the precursor β-alanine, but is also apparently due to the increased rate of carnosine synthesis. It should be noted that the formation of radiolabeled carnosine (Fig. 3) is expressed as a percentage of the total radioactivity in the cell extract

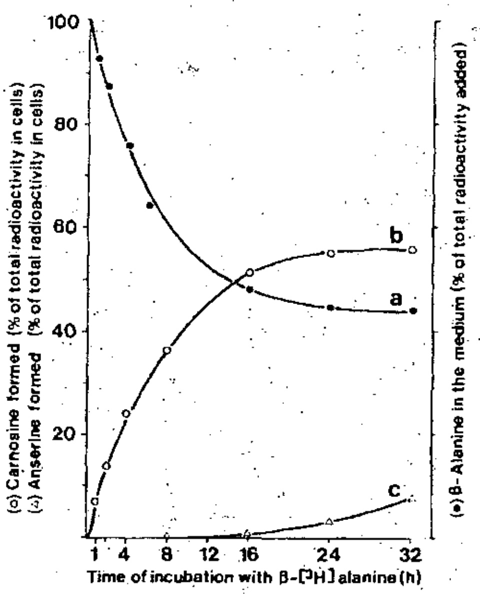

Fig. 2. Time course of β-alanine uptake and synthesis of carnosine and anserine by muscle cells in primary culture. Muscle cells cultured in growth medium for 5 days were exposed to radiolabeled β-alanine (2 µCi) for the time periods indicated. Extracts from the cells and the medium were prepared and resolved by cation-exchange chromatography on an Aminex Q-15 S column. Aliquots (500 µl) of the fractions containing β-alanine, carnosine or anserine were used for scintillation counting. The radioactive β-alanine recovered from the medium (●) is expressed relative to the total radioactivity added to the medium. The formation of radiolabeled carnosine (O) or anserine (△) is expressed relative to the total radioactivity recovered from the cell extract.

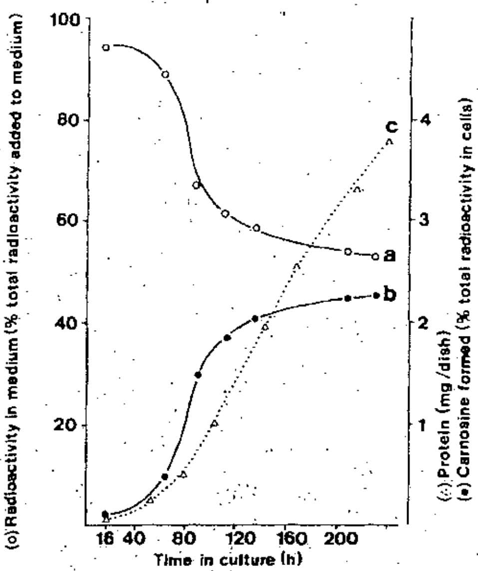

Fig. 3. Uptake of β-alanine and synthesis of carnosine by muscle cells as a function of time in culture. After plating, the cells were maintained in growth medium for the time periods indicated and were exposed to 2 µCi radiolabeled β-alanine/plate (60-mm diameter). After a 16-h incubation, aliquots of the medium were measured for radioactivity and extracts of the cells were prepared as described in the Materials and Methods section. The cell extract was fractionated by ion exchange chromatography on Aminex Q-15 S and aliquots (500 µl) of the fractions containing radiolabeled carnosine were measured for radioactivity. The radioactivity recovered from the medium (O) is expressed relative to the total radioactivity added. The formation of radiolabeled carnosine (●) is expressed relative to the total radioactivity recovered from the cell extract. The protein content (△) is also indicated.

and thus already accounts for the increased availability of the precursor. Interestingly, this increase in β-alanine uptake and carnosine synthesis coincided in time with the morphological differentiation of the myoblast to form multinucleated myotubes (Fig. 4).

DISCUSSION

Primary cultures from embryonic chick pectoral muscle were used in this study since the pectoral muscle of adult animals is a very rich source of carnosine and anserine [18] and, also, since these cultures are well-established model systems for studying the processes of muscle cell differentiation [19].

The results of the present study demonstrate that carnosine and related peptides are actively synthesized by muscle cells in primary culture and argue against the intriguing hypothesis that these peptides might only represent stored products which are actually synthesized by other cellular components of skeletal muscle. The observation, that there is no significant uptake of carnosine by muscle cells in culture also argues against the storage hypothesis (after incubation with radiolabeled carnosine for 24 h less than 3% of the radioactivity added to the medium could be recovered from the cell extract; data not shown).

After synthesis, the peptides accumulate considerably inside the cells and only small amounts are found in the culture medium. This observation is in agreement with the high concentrations of carnosine and anserine found in some skeletal muscles and may indicate an intracellular function for these peptides. Their physiological role, however, still remains unknown. The suggested but not yet verified function of carnosine as a physiological buffer substance [8] would not provide any explanation for the synthesis of anserine, a substance with comparable buffer capacity which is mainly formed via the methylation of preformed carnosine by carnosine N-methyltransferase [20], a reaction requiring S-adenosyl methionine and thus 1 mol of ATP as energy.

With muscle cells in culture, the time course of dipeptide formation (Fig. 2) also suggests that anserine is formed as a secondary metabolite and not directly by the condensation of β-alanine with 1-methylhistidine, a possible alternative pathway since biochemical studies clearly demonstrated that all ω-aminoacyl amino acids identified so far could be synthesized by one enzyme, carnosine synthetase [9]. In agreement with these biochemical studies, the synthesis of homocarnosine and homoanserine by muscle cells in culture could be demonstrated, although these peptides are exclusively found in the central nervous system due to the restricted availability of the precursor, γ-aminobutyric acid.

Surprisingly, the formation of other dipeptides could not be observed in these cultures whereas with glial cells and the C6 glioma cell-line the synthesis of β-alanyl-lysine and β-alanyl-ornithine, as well as the corresponding synthesis of γ-aminobutyric-acid-containing peptides, could be demonstrated [13, 14].

Other differences are also obvious. First of all, there is considerably more carnosine synthesized by the muscle cells in culture than by the glioma or glial cells. Furthermore, the rapid degradation of β-alanine and γ-aminobutyric acid with

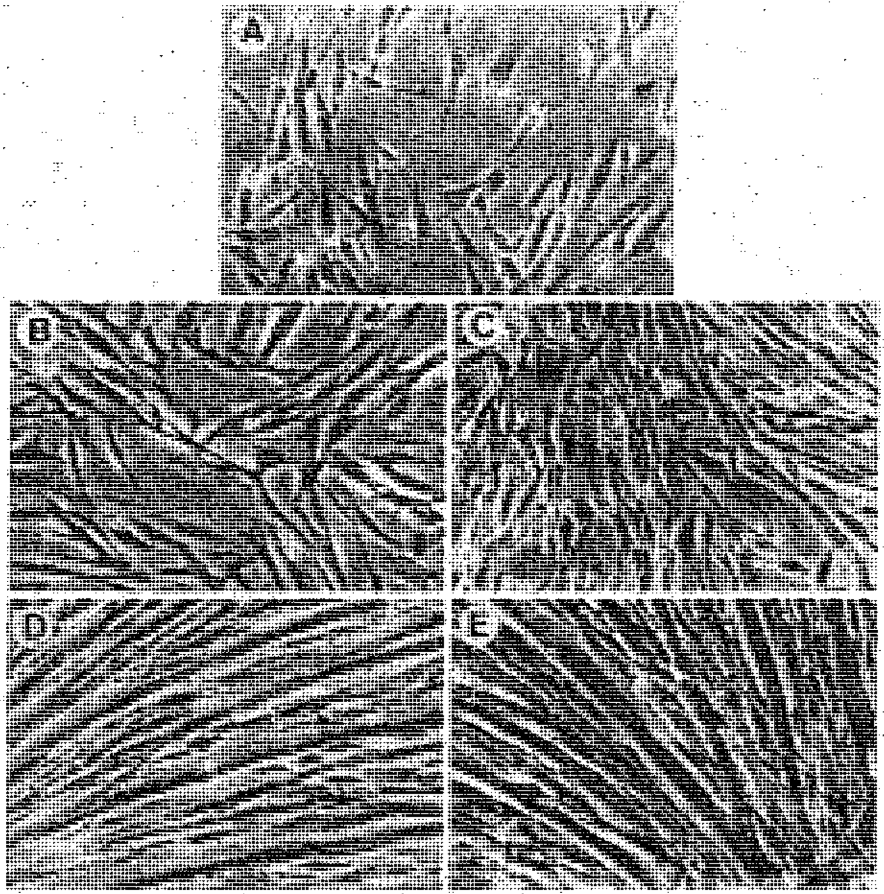

Fig. 4. Morphological changes of primary muscle cells from embryonic chick pectoral muscle. After plating, the cells were maintained in growth medium as described in the Materials and Methods section. The photographs were taken after 1 (A), 2 (B), 3 (C), 4 (D) and 7 (E) days in culture.

the formation of acidic products was observed with glial and glioma cell cultures and the synthesis of γ-aminobutyric-acid-containing dipeptides could only be observed in the presence of appropriate γ-aminobutyric-acid-transaminase inhibitors. In contrast, with muscle cells no radiolabeled acidic degradation products were found, indicating that muscle cells do not contain transaminase activity. In addition, with muscle cells in culture only small amounts of carnosine and related peptides were found in the culture medium and no significant uptake of these peptides could be observed. In contrast, with astroglia-rich primary cultures the rapid release of newly synthesized carnosine into the culture medium, as well as the rapid uptake by a dipeptide-specific transport system [21], could be demonstrated.

In astroglia-rich cultures, the coincidence of morphological differentiation and the depression of carnosine synthetase activity evoked by agents that directly or indirectly activate cyclic AMP-dependent protein kinases suggested the possibility that carnosine synthesis might be related to the differentiation of glial cells [16]. With muscle cells, the observed increase in carnosine synthetase activity also appears to coincide with the morphological differentiation of these cells, namely the fusion of mononucleated myoblasts to form multinucleated myotubes. However, since the proliferation of myoblasts still proceeds under the culture conditions used, further studies are required to delineate this relationship.

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EXHIBIT S

Eur. J. Biochem. 225, 617–623 (1994)

Transport of β-alanine and biosynthesis of carnosine by skeletal muscle cells in primary culture

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Uptake of β-alanine and synthesis of carnosine (β-alanyl-histidine) could be demonstrated in primary cell cultures derived from embryonic chick pectoral muscle. Concomitant with the morphological changes, cessation of cell division and the induction of creatine kinase, a rapid increase in the rate of β-alanine uptake and also in the rate of carnosine synthesis could be observed. The uptake of β-alanine is sodium and chloride dependent and obeys Michaelis-Menten kinetics with K_m values of about 40 µM that are essentially identical for myoblasts and myotubes. In contrast, V_{max} increases considerably during differentiation. The β-alanine transport system is highly specific for β-amino acids and exhibits a substantial anion dependency ($Cl^- > J^- > CSN^- > SO_4^{2-}$). Stoichiometric studies suggest that the transport of one β-alanine molecule involves two sodium ions and one chloride ion. This ratio is not altered by the process of cell differentiation.

Carnosine (β-alanyl-histidine) and structurally related peptides (ω-aminoacyl amino acids) are well-known constituents of excitable tissues [1, 2]. In some muscles of mammals and birds, these peptides even represent the major nonproteinaceous constituents. Since β-alanine is predominantly synthesized by the liver as a final metabolite of the pyrimidine bases uracil and thymine but not by muscle cells directly [3], muscle cells must be equipped with a highly efficient transport system for this amino acid. So far, however, uptake of β-alanine from the peripheral circulation has been extensively studied in kidney [4], brain [5] and, to some extent, also in other tissues [6, 7], but surprisingly not yet in skeletal muscle. Here, we report on the uptake of β-alanine and the synthesis of carnosine by primary cultures of chick pectoral muscle.

MATERIALS AND METHODS

Ham's F12 nutrient mixture and horse serum were obtained from Gibco. Cells were grown in cell-culture dishes (Nunc) or flasks (Falcon). 120 Ci/mmol β-[3-^{3}H(N)]alanine and 86.7 Ci/mmol 3-O-[methyl-^{3}H]methyl-D-glucose were purchased from Du Pont de Nemours and 28.4 Ci/mmol [6-^{3}H]thymidine from Amersham Buchler. Insulin, collagenase, hyaluronidase, calf thymus DNA, chloride (choline chloride), cytosine 1-β-D-arabino-furanoside (cytosine arabinoside), 3-O-methyl-D-glucopyranose and phloretin were from Sigma. Transferrin, bovine serum albumin, phosphoglycerate kinase, hexokinase, glucose-6-phosphate dehydrogenase, creatine phosphate, ADP*, NADP*, P^1,P^5-diadenosine-5′-pentaphosphate, N-acetyl-L-cysteine, NADH* and glycerate-3-phosphate were obtained from Boehringer Mannheim. Trypsin and Dowex 50 WX2 were from Serva Feinbiochemica. Bisbenzimide H 33258 fluorochrome was purchased from Calbiochem-Nova-biochem. Scintillator Hydroluma (J. T. Baker) was used for scintillation counting. Glass-fiber filters GF51 were obtained from Schleicher & Schuell. All solvents used were from Merck. Embryo extract was prepared according to the procedure of Konigsberg [8].

Cell culture

Primary cultures of myoblasts were isolated from the pectoral muscle of 11-day-old chick embryos. The muscle tissue obtained from 10 embryos was digested with 5 ml Puck's saline D2 solution (137 mM NaCl, 5.5 mM KCl, 0.22 mM KH_2PO_4, 0.168 mM Na_2HPO_4, 0.11 mM $CaCl_2$ and 5.5 mM glucose) containing 0.25% collagenase and 0.025% trypsin. After 15 min at 37°C, the cell suspension was passed through a 125-µm nylon mesh into 10 ml cold growth medium (Ham's F12 containing 15% horse serum and 5% embryo extract). The cell suspension was centrifuged at $100\times g$ for 5 min, the supernatant was aspirated and the cell pellet dispersed in 20 ml fresh growth medium. The cell suspension was then transferred to a 250-ml culture flask to allow fibroblast attachment. After 30 min, unattached cells were recovered, counted and plated onto gelatine-coated culture dishes at a density of 8×10^5 cells/plate or 3×10^5 cells/plate, 90 mm or 60 mm in diameter, respectively. The cells were grown at 37°C in a 5% CO_2-enriched atmosphere of humidified air. After 24 h, the growth medium was replaced with serum-free medium (Ham's F12, containing 1 mg/ml bovine serum albumin, 5 µg/ml transferrin, 2.5 mM $CaCl_2$ and 50 nM Na_2SeO_3). This point in time was defined as time zero. The cells grown in serum-free medium were either maintained in the same medium or challenged to differentiate by adding 10 µg/ml insulin to the serum-free medium. The latter cultures were exposed additionally to 5 µM cytosine arabinoside to obtain cultures enriched for myotubes. For all cultures, half of the medium was replaced daily with fresh medium.

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Uptake of β-alanine

The cells, cultured in 60-mm Petri dishes, were incubated for 30 min in 2 ml of the corresponding medium or Hepes/Tris buffer containing 12 µCi/ml [^{3}H]β-alanine (10 µM; specific radioactivity 1.2 Ci/mmol). The Hepes/Tris buffer consisted of 10 mM Hepes adjusted to pH 7.2 with Tris base and contained 10 mM glucose, 2.5 mM $CaCl_2$, 3.0 mM KCl, 0.6 mM $MgCl_2$ and 145 mM NaCl or other salts as specified. After incubation, the medium was removed and the cells were washed three times with 5 ml portions of ice-cold 150 mM NaCl and 10 mM sodium phosphate, pH 7.2 (NaCl/P_i). After lysis by 0.5 ml 1 M NH_4OH in 0.2% Triton X-100, the cells were scraped off the plate and the dish was washed with 0.5 ml of the same solution. The solutions were combined and aliquots of 250 µl were counted for radioactivity after addition of 10 ml Hydroluma.

Determination of carnosine synthesis

The cells grown for the time periods indicated were incubated in fresh culture medium containing 12 µCi/ml radiolabeled β-alanine (10 µM; specific radioactivity 1.2 Ci/mmol). After incubation, the medium was aspirated and the cells were washed three times with 5 ml NaCl/P_i. The washed cells were lysed by adding 1.5 ml methanolic acetic acid (2 M acetic acid in 60% methanol) and scraped off the plates. After rinsing the plates twice with 1.5 ml the same solution, the extract and the washings were combined. The samples were analysed by cation-exchange chromatography on Dowex 50 WX2 resin as described previously [9].

Creatine kinase assay

Cultures from Petri dishes (90 mm in diameter) were washed three times with 5 ml cold NaCl/P_i. The cells were scraped off the plates, centrifuged and suspended in 200 µl NaCl/P_i containing 20 mM *N*-acetylcysteine. The cell suspension was sonicated by use of a Branson Sonifer W-250 equipped with the microtip and operated at 40% for 15 s. The homogenate was centrifuged for 1 min at $10000\times g$ and the resulting supernatant was used to determine creatine kinase activity by the method of Bergmeyer et al. [10] as modified by Gerhardt et al. [11]. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 nmol NADPH/min at 30°C.

Glyceraldehyde-3-phosphate dehydrogenase assay

The cell extracts were prepared as described for the creatine kinase assay except that 100 mM triethanolamine · HCl, pH 7.6, was used as buffer. The enzymic activity was determined photometrically as described by Bergmeyer et al. [12]. One unit enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 nmol NAD^+/min at 30°C.

Incorporation of [6-^{3}H]thymidine into DNA

Cells grown on dishes 60 mm in diameter were incubated for 1 h in 2 ml medium containing 1 µCi/ml [6-^{3}H]thymidine. The cells were washed three times with 5 ml cold NaCl/P_i, then scraped off the plates in 3 ml of the same buffer. After sonication and addition of 30 µg calf-thymus DNA, 3 ml trichloroacetic acid (20% in 0.1 M sodium pyrophosphate) was added. The precipitated material was collected after 30 min on glass-fiber filters and washed three times with 5 ml 1% trichloroacetic acid before the radioactivity was determined by scintillation counting using 10 ml Hydroluma.

Measurement of intracellular water space

The amount of the non-metabolizable hexose, 3-*O*-methyl-D-glucose, taken up into the cells at equilibrium was determined in order to calculate the cell volume according to Kletzien [13].

Protein and DNA determination

Protein was determined by the Lowry method as modified by Peterson [14] using bovine serum albumin as standard. DNA was quantified fluorimetrically using H 33258 fluorochrome and calf thymus DNA as standard [15].

Calculations

Data are expressed as means ± SEM of at least three separate determinations. Eadie-Hofstee plots are least squares fits to the data. The fits to the Hill-type equation were according to Fukuhara and Turner [16].

RESULTS

Cell culture

The cell cultures from chick pectoral muscle were prepared by following the well established procedure described by Konigsberg [8]. After plating and culturing for 24 h in serum and embryo extract containing growth medium, the cultures consisted mainly of cells which express the morphology of mononucleated myoblasts (Fig. 1A). When the growth medium was replaced by serum-free medium, the cells remained mononucleated and the sparse small myotubes which were visible on day 1 and day 2 (Fig. 1A and B) were displaced by proliferating cells (Fig. 1C and D). In contrast, when the serum-free medium was supplemented with insulin (10 µg/ml), the myoblasts progressively expressed the differentiated morphology characterized by extensive fusion of the cells to form thick, multinucleated myotubes (Fig. 1E, F and G). In order to eliminate all replicating cells, the medium was supplemented additionally with 5 µM cytosine arabinoside 18 h after the switch to the insulin-containing serum-free medium. Thus, the cultures were highly enriched for myotubes.

Characteristics of the β-alanine uptake system

When the cultures were incubated with 10 µM [^{3}H]β-alanine, a linear relationship of β-alanine uptake with time could be observed (up to 120 min for the myotube cultures and up to 5 h for myoblasts and the undifferentiated cells). As shown in Table 1, the uptake of radiolabeled β-alanine was strongly inhibited by unlabeled β-alanine and by the pseudo β-amino acid taurin. The uptake of radiolabeled β-alanine was also markedly affected by γ-aminobutyric acid but not by L-alanine and glycine. Furthermore, no significant difference was noticed when the uptake of β-alanine was determined in Hepes/Tris buffer or in serum-free medium containing high concentrations of all L-α-amino acids.

The effect of β-alanine concentration on the β-alanine flux was further analyzed with myotube and myoblast cul-

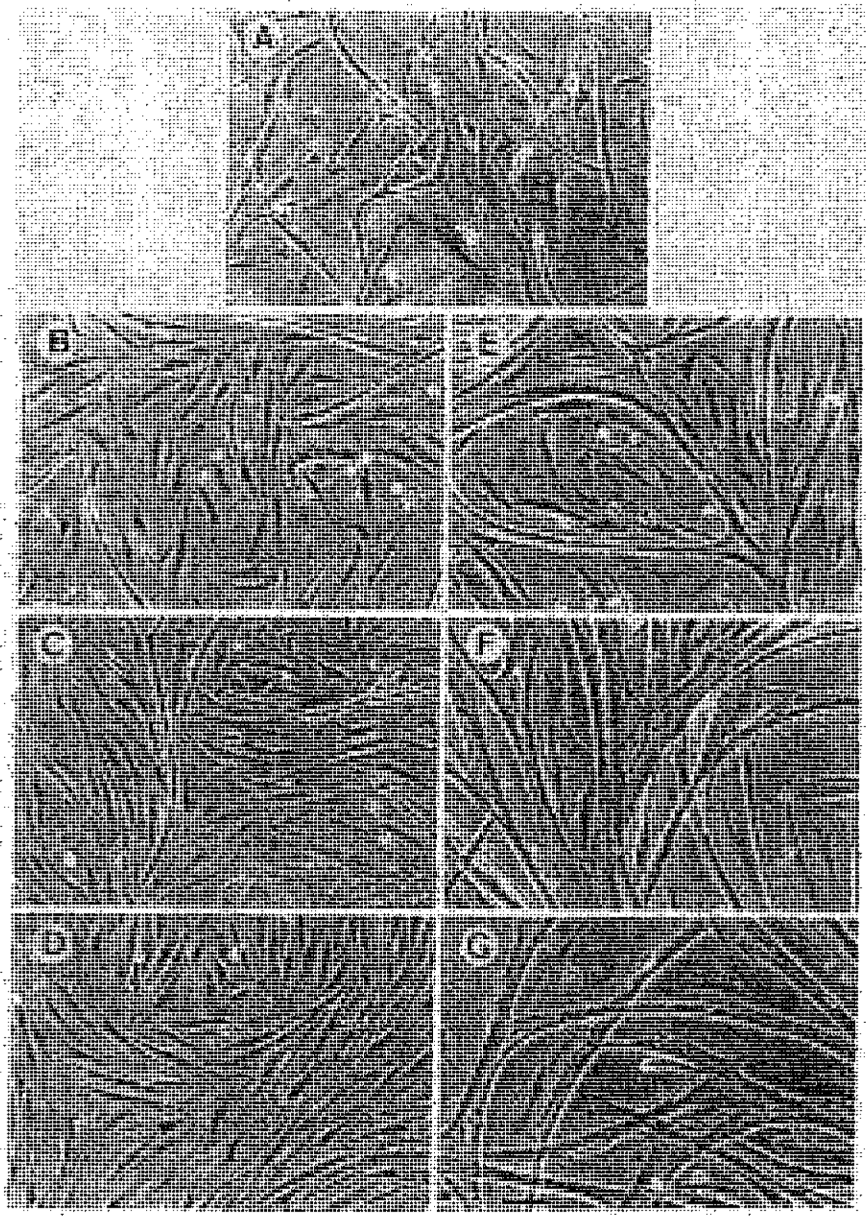

Fig. 1. Morphological changes of primary muscle cells from embryonic chick pectoral muscle. After plating, the cells were maintained for 24 h in growth medium (A, day 1) and were then maintained in serum-free medium (B, C, D; day 2,3 and 4, respectively) or in serum-free medium supplemented with 10 µg/ml insulin and 5 µM cytosine arabinoside which was added 18 h later (E, F, G; day 2,3 and 4, respectively). Bar corresponds to 75 µm.

tures (Fig. 2). The results are illustrated as an Eadie-Hofstee plot. The linearity of the plots indicate that the data fit well to the Michaelis-Menten equation and thus are consistent with the existence of a single β-alanine transport system. Least-square fits of these plots yield K_m values of about 40 µM that are almost identical for both cultures. With respect to the V_{max} values, however, considerable differences are noticed between myoblasts and myotube cultures (Fig. 2).

Ion dependency

The transport of β-alanine is strongly affected when sodium chloride is replaced by choline chloride and significantly reduced when chloride is replaced by other anions (Table 2).

For further analysis, the uptake of β-alanine was studied at different concentrations of Na^+ whereby isoosmolarity was secured by addition of choline chloride. With Na^+ concentrations of 25–145 mM, a linear relationship was obtained when the data were expressed by a Hill-type plot. Hill coefficients of $n = 1.8$ ($r = 0.942$) and 2.0 ($r = 0.995$) could be calculated for myoblasts and myotubes, respectively (Fig. 3).

To study the chloride dependency in more detail, NaCl was replaced by sodium-D-gluconate. With chloride concentrations of 25–145 mM the uptake of β-alanine followed simple kinetics and the Eadie Hofstee plots revealed a linear relationship (Fig. 4) indicating a 1:1 stoichiometry of chloride to β-alanine. Again, this ratio was independent of the processes of differentiation.

Table 1. Effects of some substances on the initial rate of [^{3}H]β-alanine uptake by myotube cultures. The compounds (final concentration 100 µM) were tested for their ability to inhibit the uptake of 10 µM radiolabeled [^{3}H]β-alanine as described in Materials and Methods. Incubation time, 30 min. The 100% control value equals the uptake of 4.53 pmol β-alanine · min^{-1} · µg DNA^{-1}.

Test substance	Relative uptake
None (control)	100
β-Alanine	33 ± 2.7
Taurine	27 ± 3.3
γ-Aminobutyric acid	42 ± 2.1
L-Alanine	92 ± 3.9
Glycine	91 ± 1.0

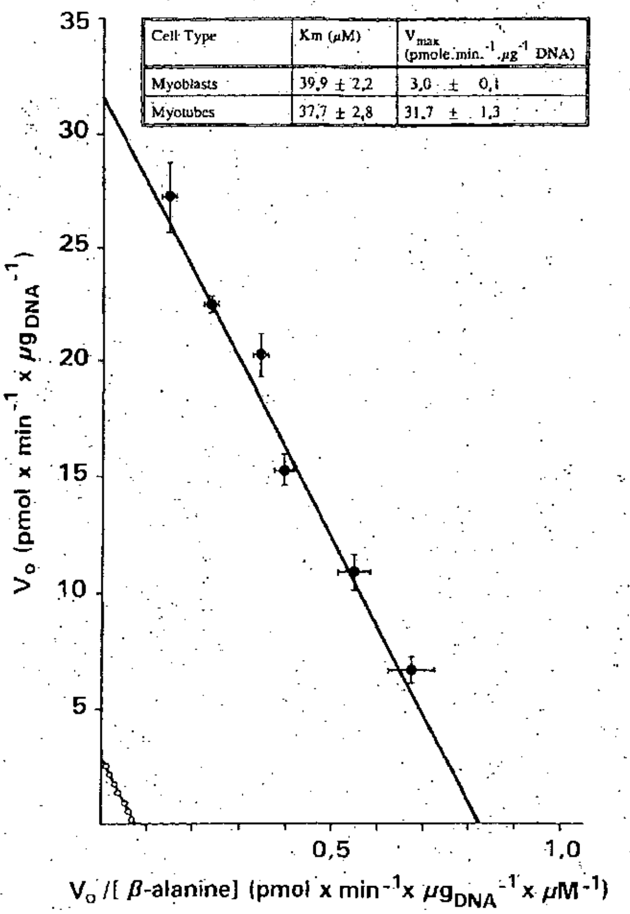

Fig. 2. Effect of β-alanine concentration on the initial rate of β-alanine uptake. The cells were cultured as described in the legend to Fig. 1 and used either immediately after the switch to serum-free medium (myoblasts) or after cultivation for 72 h under differentiating conditions (myotubes), respectively. The uptake of [^{3}H]β-alanine in the presence of varying amounts of unlabeled β-alanine was determined after incubation for 30 min as described in Materials and Methods. Eadie-Hofstee plots in which the initial velocity v_o was plotted against v_o/[β-alanine] are presented for myoblasts (○; r = 0.985) and myotubes (●; r = 0.989).

Uptake of β-alanine as a function of muscle cell differentiation

Since we noticed a considerable difference in the rate of β-alanine uptake by myotube and myoblast cultures, we also

Table 2. Effects of ions on the initial rate of β-alanine uptake by myotube cultures. The cells were incubated for 30 min with radiolabeled β-alanine (10 µM) either in control medium containing 145 mM NaCl or in media in which NaCl was replaced by equimolar concentrations of other salts. The uptake of β-alanine was determined as described in Materials and Methods.

Test substance	v_0	Uptake
	pmol · min^{-1} · µg DNA^{1}	%
Control (NaCl)	4.50 ± 0.06	100
Choline chloride	0.12 ± 0.01	2.6
Sodium iodide	1.32 ± 0.04	29
Sodium thiocyanate	0.97 ± 0.02	22
Sodium sulfate	0.45 ± 0.04	10

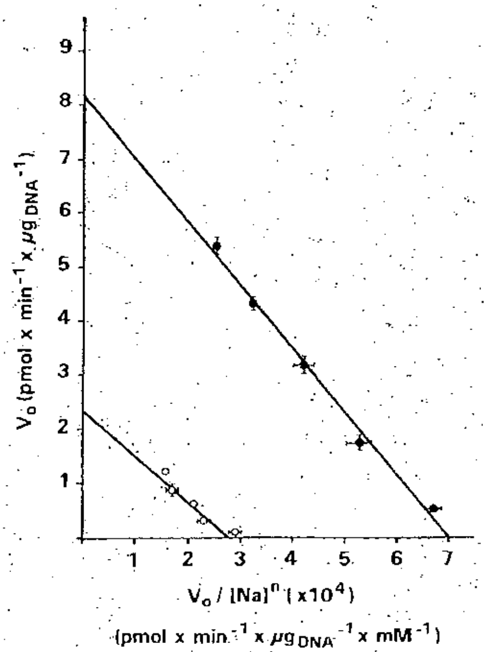

Fig. 3. Dependence of β-alanine uptake on Na$^+$ concentration. Muscle cells grown for 24 h in growth medium and switched to serum-free medium (myoblasts) or cultured additionally for 72 h under differentiating conditions (myotubes) were incubated in Hepes/Tris buffer, pH 7.2, containing [^{3}H]β-alanine, 145 mM Cl$^-$ and different concentrations of Na$^+$, whereby isoosmolarity was maintained by substituting NaCl with choline chloride. Uptake of [^{3}H]β-alanine (10 µM; specific radioactivity 1.2 Ci/mmol) was measured after incubation for 30 min. For details, see Materials and Methods. Hill-type plots are presented for myoblasts (with n = 1.8; ○, r = 0.942 and myotubes n = 2; ●, r = 0.995).

determined the initial rate of β-alanine flux over the periods of cellular differentiation. Within the first 7 h after the switch from the growth medium containing serum and embryo extract to serum-free medium, a steep increase in the initial rate of β-alanine uptake could be observed for both cultures (Fig. 5A). Thereafter, however, the initial rate of β-alanine uptake only increased progressively when the cells were kept under differentiating conditions (Fig. 5A, curve a). This increase correlates well with the morphological changes (Fig. 1), the decrease of [^{3}H]thymidine incorporation into DNA (Fig. 5B, curve a) and also with the changes in the

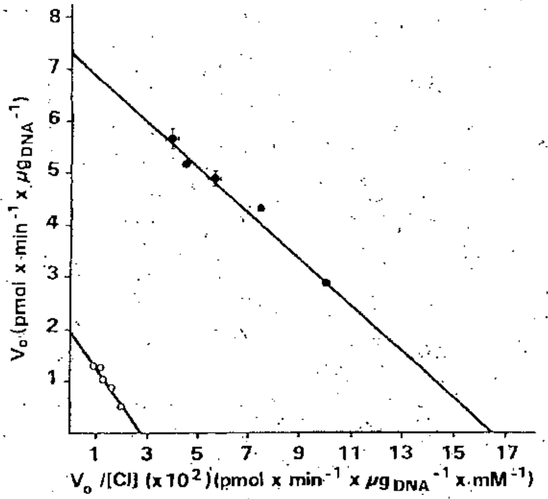

Fig. 4. Dependence of β-alanine uptake on Cl^- concentrations. As described in the legend to Fig. 3, myoblast and myotube cultures were incubated for 30 min in Hepes/Tris buffer, pH 7.2, containing [^{3}H]β-alanine and different concentrations of Cl^-, made by substituting NaCl isoosmotically with sodium gluconate. Eadie-Hofstee plots in which the initial velocity v_o was plotted against v_o/[Cl^-] are presented for myoblasts (○; $r = 0.945$) and myotubes (●; $r = 0.979$).

activity of creatine kinase (Fig. 5C, curve a), an early marker of myogenesis. In contrast, when kept in serum-free medium without insulin and cytosine arabinoside, the cells continue to proliferate and to incorporate [^{3}H]thymidine into DNA (Fig. 5B, curve b), but neither exhibit an increase in β-alanine uptake (Fig. 5A, curve b) nor in creatine kinase activity (Fig. 5C, curve b). Regardless of the culture conditions used, a significant difference in the activity of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase could not be observed (11.6 ± 0.83 nmol NAD* · min^{-1} · μg^{-1} DNA for the differentiated cells after 72 h in culture versus 9.1 ± 0.71 nmol NAD* · min^{-1} · μg^{-1} · DNA for undifferentiated cells also cultured for 72 h).

Biosynthesis of carnosine

Concomittant with cellular differentiation, we also observed a significant increase in the amount of carnosine that could be isolated from the extracts of cells which were kept under differentiating culture conditions (Fig. 5D, curve a). This increase was not observed with extracts from non-differentiated cells (Fig. 5D, curve b). This difference could also be noted when the formation of radiolabeled carnosine was expressed relative to the total radioactivity in the cell extract in order to account for the increased availability of the precursor (data not shown). To evaluate this aspect further, we also determined the cell volume using the method described by Kletzien et al. [13] which measures the amount of the non-metabolizable hexose 3-O-[methyl-^{3}H]choline-D-glucose that is taken up at equilibrium. By this method, cell volumes of 5.66 ± 0.34 μl/mg protein and 6.79 ± 0.27 μl/mg protein were determined for myotubes and myoblasts, respectively. Thus, it could be calculated that, within 1 h, the intracellular β-alanine concentrations reached values of 1180 ± 40 μM in myotubes and 206 ± 20 μM in myoblasts. Since, with purified carnosine synthetase from chick pectoral muscle, a K_m value of about 40 μM has been determined for

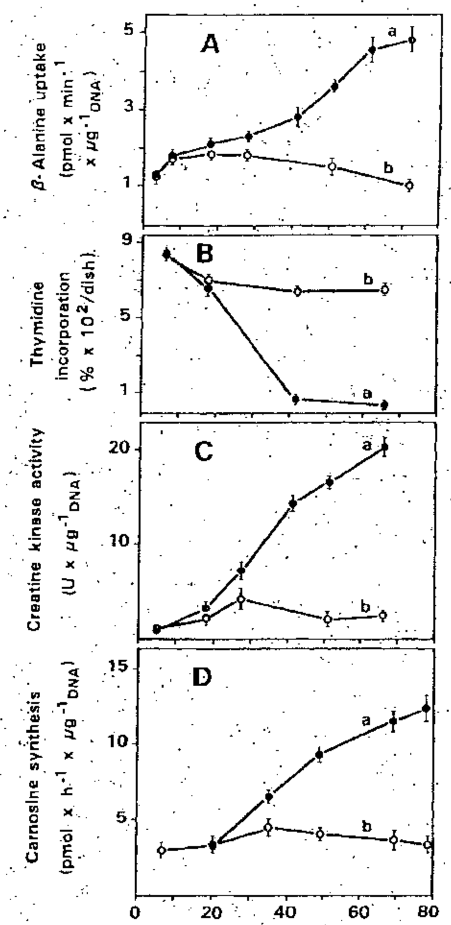

Fig. 5. Changes in the rate of β-alanine uptake (A), the incorporation of [^{3}H] thymidine (B), the activity of creatine kinase (C) and in the rate of carnosine synthesis (D) during the cultivation of muscle cells under differentiating conditions (lines a) and non-differentiating conditions (lines b). Muscle cells were grown for 24 h as described in Materials and Methods. The growth medium was then replaced (time zero) either by serum-free medium (○; non-differentiating conditions) or by serum-free medium supplemented with 10 μg/ml insulin and 18 h later additionally with 5 μM cytosine arabinoside (●; differentiating conditions). After the indicated periods of time, the uptake of β-alanine, synthesis of carnosine, creatine kinase activity and [^{3}H]thymidine incorporation was determined as described in Materials and Methods.

β-alanine [17, 18], it can be assumed that for the synthesis of carnosine, saturating β-alanine concentrations were reached within a relatively short period of time compared to the incubation times (1 h for myotubes and 5 h for myoblasts and undifferentiated cells) that were used to determine the amount of carnosine formed. These incubation times were chosen to reach comparable β-alanine concentrations in both cell types (≈ 250 nmol β-alanine/μg DNA). Under the experimental conditions used, preceding experiments had already demonstrated that the uptake of β-alanine followed a linear relationship with time. Therefore, these results indicated that the availability of the precursor β-alanine is obviously not the limiting factor and strongly suggested that the difference in the amount of carnosine which could be isolated from these cultures reflect the differences in the rate of carnosine synthesis.

DISCUSSION

Primary cultures from embryonic chick pectoral muscle were used in this study since pectoral muscle from adult animals is a very rich source of carnosine and anserine [2] and also since these cultures are well-established model systems for studying the processes of muscle cell differentiation [19]. Low-serum-(2%)-containing media [20] or serum-free media supplemented with transferrin and supraphysiological concentrations of insulin [20, 21] have been successfully used to stimulate the differentiation of myoblast cultures in the presence of cytosine arabinoside at concentrations which completely block DNA synthesis [21]. Under these conditions, we observed that the increase in the rate of β-alanine uptake and carnosine biosynthesis correlates in time with the morphological changes of the cells, the decrease of [^{3}H]thymidine incorporation into DNA and the rapid enhancement in the activity of creatine kinase, an established early marker of myogenic differentiation [22, 23].

The characteristics of the skeletal muscle β-alanine transport system are comparable, on the whole, with the characteristics of other β-amino acid transporters. Like the β-alanine and taurine uptake systems that have been studied with renal brush-border membrane vesicles [4, 24], synaptosomes [5] and hepatic basolateral plasma membrane vesicles [25], the β-alanine transport of muscle cells is highly specific for β-amino acids and is strongly dependent on sodium and chloride. The hyperbolic dependency of the initial rate of β-alanine uptake on the chloride concentration indicates a 1:1 stoichiometry of Cl^-/β-alanine that is not altered by the cellular differentiation of muscle cells. With regard to the sodium dependency, the stoichiometric analysis indicates that a minimum of two sodium ions are required for the transport of one molecule of β-alanine. The Hill-coefficient values of $n = 1.8$ for myoblasts and $n = 2$ for myotubes were obtained when the β-alanine flux was analyzed within Na^+ concentrations ranging over 25–145 mM. In contrast, during the differentiation of astrocytes and neurons in primary culture, a change in the Hill coefficients from 1 to 2 has been observed for the sodium-dependent uptake of γ-aminobutyric acid by these cells. During the cellular differentiation of muscle cells, there is apparently also no change in the affinity of the transporter for β-alanine. For myoblasts and myotubes, almost identical K_m values (about 40 μM) were found. With regard to the V_{max} values, however, a tenfold difference was noticed between myoblasts and myotubes. These data indicate that the amount of the transporter protein increased considerably during the process of differentiation while the quality of the protein remained unaltered. Similar observations have also been made with other systems, e.g. for the high-affinity uptake of γ-aminobutyric acid by astrocytes and neurons in primary culture [26].

The kinetical data of the β-alanine transport by muscle cells also compare well with the data reported for other systems such as the high-affinity low-capacity β-alanine uptake system of renal [4] and intestinal [7] brush border membranes.

Due to the high efficiency of this transport system, β-alanine readily reaches intracellular concentrations exceeding by far the K_m value that has been determined for β-alanine with purified carnosine synthetase from chick pectoral muscle [17, 18]. Although, in cell culture, the synthesis of carnosine is secondary to the uptake of β-alanine, we conclude that, under our experimental conditions, the amount of carnosine which could be isolated from the cell extracts rather reflects the activity of carnosine synthetase than the uptake of β-alanine. This notion is supported by the observation that the rate of carnosine synthesis considerably increased concomitantly with the process of muscle-cell differentiation. This increase in carnosine synthesis could be influenced by a variety of factors. One likely interpretation would be that the increased enzymic synthesis might be due to an increase in the amount of carnosine synthetase and/or the appearance of an isoenzyme with different catalytic properties. This hypothesis cannot be tested by cell-culture studies and, unfortunately, also not with extracts prepared from these cells since, in crude cell extracts, carnosine synthetase activites are very low due to a variety of inhibitory substances and ATP-hydrolyzing activities (unpublished results). Verification of this interpretation and others have to await the further purification and characterization of carnosine synthetase. Such hypotheses in relation to the biological function of carnosine and related peptides deserve further inquiry.

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EXHIBIT T

Creatine ingestion favorably affects performance and muscle metabolism during maximal exercise in humans

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Department of Physiology and Pharmacology, University of Nottingham Medical School, Queen's Medical Center, Nottingham NG7 2UH, United Kingdom; and Department of Clinical Chemistry, Karolinska Institute, Huddinge University Hospital, S-141 86 Huddinge, Sweden

Casey, A., D. Constantin-Teodosiu, S. Howell, E. Hultman, and P. L. Greenhaff. Creatine ingestion favorably affects performance and muscle metabolism during maximal exercise in humans. *Am. J. Physiol.* 271 (*Endocrinol. Metab.* 34): E31–E37, 1996.—Nine male subjects performed two bouts of 30-s maximal isokinetic cycling before and after ingestion of 20 g creatine (Cr) monohydrate/day for 5 days. Cr ingestion produced a 23.1 ± 4.7 mmol/kg dry matter increase in the muscle total creatine (TCr) concentration. Total work production during *bouts 1* and *2* increased by ~4%, and the cumulative increases in both peak and total work production over the two exercise bouts were positively correlated with the increase in muscle TCr. Cumulative loss of ATP was 30.7 ± 12.2% less after Cr ingestion, despite the increase in work production. Resting phosphocreatine (PCr) increased in type I and II fibers. Changes in PCr before *exercise bouts 1* and *2* in type II fibers were positively correlated with changes in PCr degradation during exercise in this fiber type and changes in total work production. The results suggest that improvements in performance were mediated via improved ATP resynthesis as a consequence of increased PCr availability in type II fibers.

phosphocreatine; adenosine 5′-triphosphate; muscle fiber types

FATIGUE SUSTAINED DURING short-term maximal-intensity exercise in humans has been associated with the inability of skeletal muscle to maintain a high rate of anaerobic ATP production from phosphocreatine (PCr) hydrolysis (6, 18, 21). Furthermore, evidence is also available to suggest that fatigue under these conditions may be attributable to an impairment of ATP production predominantly in type II muscle fibers, in which the PCr concentration is rapidly depleted (7a, 25).

Harris et al. (16) were the first to demonstrate that ingestion of 5 g creatine (Cr) monohydrate on four to six occasions each day for several consecutive days could increase the total creatine (TCr; ΣPCr and Cr) concentration of human skeletal muscle by an average of 25 mmol/kg dry matter, some 30% of which occurred in phosphorylated form as PCr. The authors went on to suggest that these changes might have a beneficial effect on exercise performance in humans, and indeed recent placebo controlled studies, using a variety of experimental models, have confirmed that dietary Cr supplementation can improve exercise performance during repeated bouts of maximal-intensity exercise (1, 5, 8, 12, 17).

To date, there has been no direct investigation of the effects of Cr ingestion on muscle metabolism during maximal-intensity dynamic exercise. However, several of the performance studies cited above found a reduction in plasma ammonia (5, 12) and hypoxanthine (1) accumulation during exercise after Cr ingestion, despite increases in muscle torque/work production. As a consequence of these findings, the ergogenic effect of Cr ingestion was attributed to possible improvements in the ability of muscle to sustain ATP rephosphorylation from ADP during exercise, which may have been achieved as a result of an increase in preexercise PCr availability, an improvement in muscle buffering capacity, and/or an acceleration of PCr resynthesis during exercise and recovery. In support of this latter hypothesis, acceleration of PCr resynthesis during recovery from intense electrically evoked isometric muscle contraction has been shown to occur after Cr supplementation (11). Whether a similar relationship exists between Cr availability and changes in metabolism during maximal dynamic exercise of short duration has yet to be ascertained.

The studies of Harris et al. (16) and Greenhaff et al. (11) also drew attention to the large interindividual variation in the change in muscle TCr concentration, which appeared to be at least partly related to the initial TCr concentration of the muscle. However, perhaps more importantly, Greenhaff et al. (11) showed that a measurable effect of Cr ingestion on PCr resynthesis during recovery from maximal-intensity exercise was only observed in individuals who demonstrated more than a 20 mmol/kg dry matter increase in muscle TCr concentration after Cr ingestion. These results suggest that a favorable effect of Cr ingestion on metabolism and performance during exercise and recovery may be critically dependent on the magnitude of the increase in muscle TCr concentration during supplementation.

The aim of the present experiment, therefore, was to perform a direct investigation of the effects of Cr supplementation on skeletal muscle energy metabolism and performance during repeated bouts of maximal exercise in humans. It was hypothesised that *1*) a relationship would exist between increases in muscle Cr availability and improvements in maximal dynamic exercise performance and *2*) that, since fatigue during maximal exercise is associated with a fall in ATP resynthesis from PCr degradation in type II muscle fibers (7a, 25), an increase in type II fiber PCr availability might improve the maintenance of force production via an effect on ATP resynthesis.

METHODS

Subjects. Nine healthy male subjects gave their written consent to take part in the present study, which was approved by the University of Nottingham Medical School Ethical

Committee. The level of fitness of the subjects was assessed by means of their training diaries. All subjects were well trained in the sense that they trained 5–6 days/wk on a regular basis, and all competed in various sports to a good club level. Mean (±SE) age, height, and weight were 27 ± 1 yr, 185 ± 2 cm, and 78 ± 2 kg, respectively. Before beginning the study, all subjects participated in a routine medical examination.

Experimental protocol. On the first visit to the laboratory, subjects were thoroughly familiarized with maximal-intensity isokinetic cycling and all procedures involved in the experiment. All subjects then reported back to the laboratory after an overnight fast on four further occasions, having been instructed to abstain from strenuous physical activity and alcohol intake for 24 h, from caffeine intake on the day of each experiment, and having maintained dietary intake to as close to normal as possible.

On the first occasion, subjects performed two bouts of 30-s maximal-intensity isokinetic cycling. Each bout of exercise was performed at 80 revolutions/min and was separated by 4 min of passive recovery during which subjects rested on a couch. This protocol was repeated on a second visit, separated from the first by ~5 days, to assess reproducibility of total work production. Subjects in whom measurements of total work production differed by >5% were recalled and were required to repeat the procedure. At the end of this period of familiarization, measurements of total work production during exercise, measured over a period of 1 wk, differed by 2.6 ± 0.5% (n = 9 subjects).

On a third visit, all subjects repeated the two bouts of maximal-intensity exercise again; however, on this occasion, muscle biopsy samples were obtained from the vastus lateralis (2) immediately before and after each bout of exercise. All biopsies were obtained from one limb that was chosen at random before the start of the study and were obtained while the subjects remained seated on the cycle ergometer, with the exception of the initial resting biopsy, which was obtained with subjects resting on a couch. Two days later, subjects proceeded to consume 20 g Cr/day (Cairn Chemicals, Chesham, UK) for five consecutive days. All subjects were instructed to consume 5 g Cr, dissolved in 250 ml of a warm-hot beverage, on four occasions at equally spaced intervals. The morning after the final day of Cr ingestion, subjects reported back to the laboratory on a final occasion and repeated the exercise procedures that they had previously performed. Likewise, muscle biopsy samples were obtained immediately before and after each bout of exercise, but on this occasion the contralateral limb was used.

Measurements of average work production per pedal revolution (J) and total work production (J/kg body mass) were obtained as described in the accompanying paper (8a). Peak work production refers to the maximum value obtained during 30 s of exercise.

Muscle sampling and analysis. Sampling and analytic procedures are described in the accompanying paper (8a). In the present study, ATP, PCr, Cr, glucose 6-phosphate (G-6-*P*), and lactate concentrations were measured spectrophotometrically in mixed muscle (14). Of the total number of individual muscle fibers dissected, the amount successfully classified as type I or type II was 88 ± 1%. Mean pooled weight of the type I and type II fibers was 21.2 ± 0.6 and 22.8 ± 0.5 µg, respectively. PCr concentrations in type I and type II fibers were measured using a fluorimetric modification of the method of Harris et al. (14).

Statistical analysis. All exercise performance data refer to n = 9 subjects. Muscle metabolite data presented in the text, Tables 1 and 2, and Figs. 1–5 before Cr ingestion refer to n = 9 subjects and post-Cr ingestion to n = 8 or 7 subjects. This is because, after Cr ingestion, it was not possible to obtain postexercise biopsy samples from one subject, and a second subject chose not to have any biopsy samples taken.

Differences in peak and total work production, and muscle metabolite concentrations (both absolute and Δ values) during each bout of exercise pre- and post-Cr supplementation were examined using repeated-measures analysis of variance (BMDP 2V/5V), with experimental condition (pre- and post-Cr) and time (number of consecutive exercise bouts) as factors. This software allows unbalanced analysis of variance to be performed on incomplete data sets without estimation of the missing data. Scheffé's test for comparing mean values was used as a post hoc test when significant interactions were detected.

Relationships between variables were examined by computing the Pearson product-moment correlation coefficient (r). Statistical significance was accepted at the 5% level (P < 0.05). Values are presented in the text, Tables 1 and 2, and in Figs. 1–5 as means ± SE.

RESULTS

Muscle torque production. Peak work production was recorded within 1.8 ± 0.2 s of exercise and is shown in Fig. 1*A*. After Cr ingestion, seven of the nine subjects showed an improvement in peak work production during *bouts 1* and *2*, but a statistical difference was not quite achieved (4.1 ± 2.0 and 3.8 ± 1.7%, respectively; P = 0.052). Figure 1*B* shows total work production (J/kg body mass) during *exercise bouts 1* and *2* before and after Cr ingestion. After Cr ingestion, total work

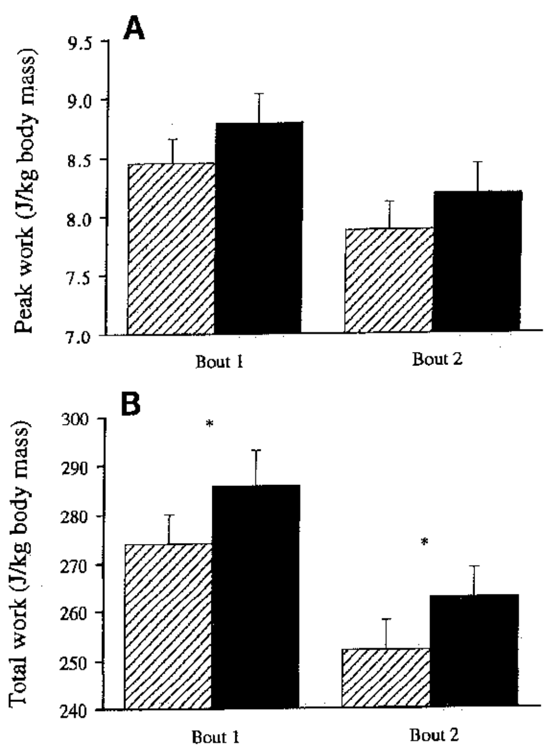

Fig. 1. Peak and total work production during 2 bouts of 30-s maximal-intensity isokinetic cycling exercise. Each bout of exercise was performed at 80 revolutions/min and was separated by 4 min of passive recovery. Values are given precreatine (hatched bars) and postcreatine (filled bars) supplementation for 5 days (4 × 5 g/day). Values represent means ± SE. *Significant differences precreatine and postcreatine supplementation (P < 0.05).

Table 1. *Mixed-muscle ATP, PCr, Cr, TCr (ΣPCr and Cr), G-6-P, and lactate concentrations measured immediately before and after 2 bouts of 30-s maximal-intensity isokinetic cycling exercise*

	Pre-Cr Ingestion				Post-Cr Ingestion			
	Pre-B1	Post-B1	Pre-B2	Post-B2	Pre-B1	Post-B1	Pre-B2	Post-B2
ATP	23.9 ± 0.4	18.7 ± 1.3*	20.7 ± 1.4	17.3 ± 1.0*	23.3 ± 0.5	18.7 ± 1.2*	19.4 ± 1.3	17.9 ± 1.3
PCr	83.9 ± 3.6	34.8 ± 4.5*	72.8 ± 3.0	24.2 ± 1.9*	92.3 ± 1.8†	35.0 ± 3.1*	75.3 ± 3.4	27.9 ± 4.5*
Cr	43.6 ± 0.9	92.4 ± 7.1*	55.0 ± 3.7	98.6 ± 3.9*	60.0 ± 3.1†	113.4 ± 8.1*†	72.2 ± 5.6†	124.9 ± 7.3*†
TCr	127.7 ± 3.4	129.8 ± 4.2	127.8 ± 4.1	122.5 ± 3.8	151.6 ± 4.3†	148.4 ± 5.5†	147.5 ± 4.5†	152.8 ± 6.8†
G-6-*P*	2.5 ± 0.9	21.6 ± 2.8*	8.4 ± 1.9	17.0 ± 2.0*	1.9 ± 0.2	20.9 ± 2.0*	8.1 ± 1.6	17.6 ± 1.5*
Lactate	4.4 ± 0.5	90.0 ± 13.0*	49.7 ± 9.8	112.7 ± 10.3*	3.9 ± 0.7	86.1 ± 12.3*	49.7 ± 9.5	115.5 ± 11.8*

Values are means ± SE in mmol/kg dry matter. PCr, phosphocreatine; Cr, creatine; TCr, total creatine; G-6-*P*, glucose 6-phosphate; B1, bout 1; B2, bout 2. Each bout of exercise was performed at 80 revolutions/min and was separated by 4 min of recovery. Values are given precreatine and postcreatine supplementation for 5 days (4 × 5 g/day). *Significant differences pre- and postexercise ($P \leq 0.01$). †Significant differences between corresponding concentrations pre- and post-Cr ingestion ($P < 0.05$).

production increased by 11.6 ± 3.1 J/kg body mass (4.2 ± 1.1%; $P < 0.05$) and 10.9 ± 2.9 J/kg body mass (4.4 ± 1.2%; $P < 0.05$) in *exercise bouts 1* and *2*, respectively. Irrespective of treatment, total work production was always greatest during the first bout of exercise ($P < 0.01$).

Mixed-muscle metabolites. Table 1 shows mixed-muscle metabolite concentrations before and after *exercise bouts 1* and *2*, pre- and post-Cr ingestion. Cr ingestion resulted in a 23.1 ± 4.7 mmol/kg dry matter (18.5 ± 3.9%; $P < 0.05$) increase in muscle TCr concentration, of which ~⅓ was in the form of PCr (8.4 ± 4.4 mmol/kg dry matter, $P < 0.05$; range −3.2 to 26.9 mmol/kg dry matter) and the remainder in the form of Cr (16.5 ± 3.4 mmol/kg dry matter, $P < 0.05$; range 3.8–27.4 mmol/kg dry matter). The individual increases in muscle TCr concentration after Cr supplementation are shown in Fig. 2, in which subjects have been numbered one to eight, according to their initial muscle TCr concentration. This figure demonstrates that interindividual variation in the increase in muscle TCr concentration was large, ranging from 6.4 to 37.8 mmol/kg dry matter. Figure 3 shows increases in muscle TCr concentration as a result of Cr supplementation with reference to cumulative changes in peak and total work production over the two exercise bouts. Numbers one to eight in Fig. 3 refer to the subjects depicted in Fig. 2. It is evident that the increase in muscle TCr concentration after Cr supplementation was positively correlated with the cumulative change in peak work production during *exercise bouts 1* and *2* ($r = 0.71$, $P < 0.05$; Fig. 3*A*). Similarly, the increase in muscle TCr concentration was positively correlated with the cumulative increase in total work production during *exercise bouts 1* and *2* ($r = 0.71$, $P < 0.05$; Fig. 3*B*). No correlation was found between changes in the preexercise PCr concentration after Cr supplementation and changes in either total work ($r = -0.013$) or peak work ($r = -0.191$) production.

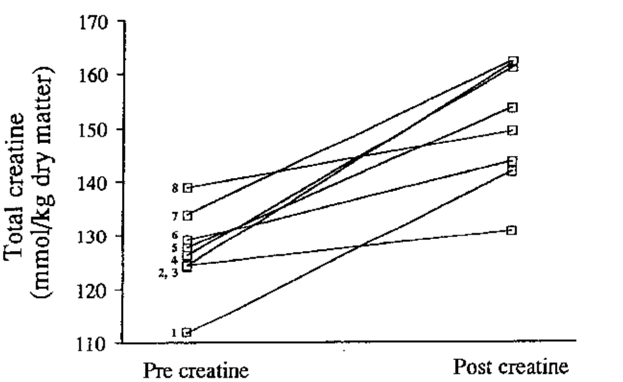

Fig. 2. Mixed-muscle total creatine (TCr; Σphosphocreatine and creatine) concentration in individual subjects precreatine and postcreatine supplementation for 5 days (4 × 5 g/day). Values represent the mean of biopsies obtained before and after 2 bouts of 30-s maximal-intensity isokinetic cycling exercise ($n = 4$) precreatine and postcreatine supplementation. Subjects have been numbered 1–8 based on their initial muscle TCr concentration.

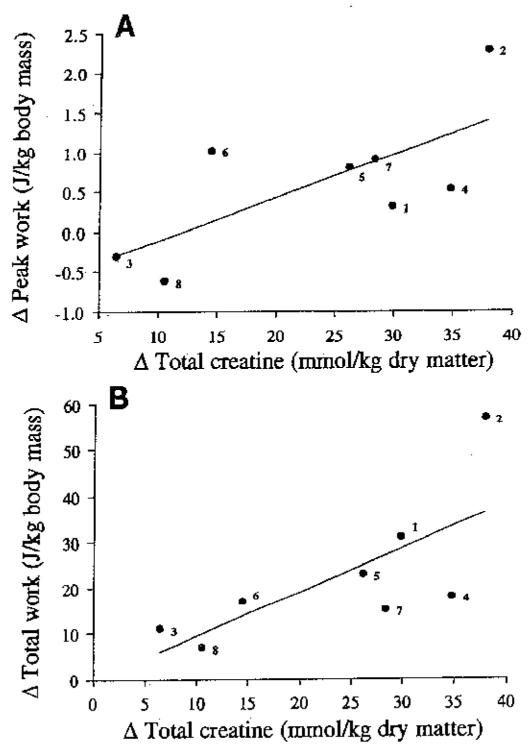

Fig. 3. Relationship between individual changes in mixed-muscle total creatine (Σphosphocreatine and creatine) concentration and cumulative changes in peak ($r = 0.71$, $P < 0.05$; *A*, $y = -0.65 + 0.05x$) and total ($r = 0.71$, $P < 0.05$; *B*, $y = -0.29 + 0.96x$) work production over 2 bouts of exercise. Values are given precreatine and postcreatine supplementation for 5 days (4 × 5 g/day). *Numbers 1–8* refer to same subjects depicted in Fig. 2.

The initial bout of exercise resulted in a marked degradation of PCr (Table 1), but no significant difference was found when comparing treatments (49.1 ± 6.6 and 57.2 ± 3.1 mmol/kg dry matter pre- and post-Cr ingestion, respectively). Furthermore, no difference in PCr resynthesis during the recovery period was found pre- and post-Cr ingestion (88.1 ± 6.1 and 81.7 ± 3.7% of the preexercise resting value, respectively). PCr degradation during the second bout of exercise was close to that observed during the first bout on both treatments, and no difference was found when comparing treatments (48.6 ± 2.9 and 47.4 ± 4.0 mmol/kg dry matter, pre- and post-Cr ingestion, respectively).

Figure 4 shows the decline in muscle ATP concentration during *exercise bouts 1* and *2* and the cumulative loss of muscle ATP over the two exercise bouts pre- and post-Cr ingestion. Mean ATP loss was numerically, but not significantly, reduced during individual exercise bouts after Cr ingestion; however, cumulative ATP loss was reduced by 30.7 ± 12.2% (8.5 ± 1.0 and 6.7 ± 1.3 mmol/kg dry matter pre- and post-Cr ingestion, respectively; $P < 0.05$), despite a concomitant increase in work production. No correlation was found between the reduction in muscle ATP loss after Cr ingestion and changes in either total work ($r = -0.395$) or peak work ($r = -0.077$) production. Furthermore, no correlation was found between changes in preexercise PCr availability and the reduction in muscle ATP loss ($r = 0.33$). ATP resynthesis during the recovery period was negligible (1.9 ± 0.6 and 0.8 ± 0.7 mmol/kg dry matter, pre- and post-Cr ingestion, respectively), and no difference in ATP concentration at the end of the recovery period was observed pre- and post-Cr ingestion (Table 1).

The initial bout of exercise resulted in approximately a 10-fold increase in muscle G-6-*P* and a 20-fold increase in muscle lactate, reflecting the highly anaerobic nature of the exercise performed. Both muscle G-6-*P* and lactate returned toward resting concentrations during recovery between exercise bouts. No differences were observed at any point when comparing G-6-*P* and lactate accumulation between treatments (Table 1).

Type I and type II muscle fiber metabolites. Table 2 shows the PCr concentration of type I and type II muscle fibers before and after *exercise bouts 1* and *2*, pre- and post-Cr ingestion. Preexercise, resting PCr increased in both type I (11.1 ± 3.7 mmol/kg dry matter; $P < 0.05$) and type II (14.1 ± 7.4 mmol/kg dry matter; $P = 0.05$) fibers after Cr ingestion. Similar to the increase in mixed-muscle TCr concentration, a large interindividual variation was observed in the change in resting PCr after Cr ingestion (type I fiber range 3.5–20.3 mmol/kg dry matter; type II fiber range −9.2 to 37.1 mmol/kg dry matter). There was also a suggestion that the PCr concentration before *exercise bout 2* increased in type I (9.6 ± 4.3 mmol/kg dry matter) and type II (12.4 ± 7.4 mmol/kg dry matter) fibers after Cr ingestion (Table 2), but a large variation was found between subjects (type I fibers −5.6 to 24.2 mmol/kg dry matter; type II fibers −9.2 to 37.8 mmol/kg dry matter).

Degradation of PCr in type I and type II fibers is shown pre- and post-Cr ingestion in Fig. 5. After Cr ingestion, mean PCr degradation in type I fibers remained unchanged during *exercise bout 1* but was greater during *exercise bout 2* ($P < 0.05$). Similarly, mean PCr degradation in type II fibers was not affected by Cr ingestion during *exercise bout 1* but was greater during *exercise bout 2* ($P < 0.05$).

The increase in PCr concentration before *exercise bouts 1* and *2* in type II fibers, after Cr supplementation, was positively correlated with both an increase in PCr degradation during exercise in this fiber type ($r = 0.78$, $P < 0.01$) and with the increase in total work production ($r = 0.66$, $P < 0.05$). No corresponding correlations were found between the change in PCr concentration before *exercise bouts 1* and *2* in type I fibers, after Cr supplementation, and changes in either PCr degradation during exercise ($r = 0.22$) or total work production ($r = 0.32$).

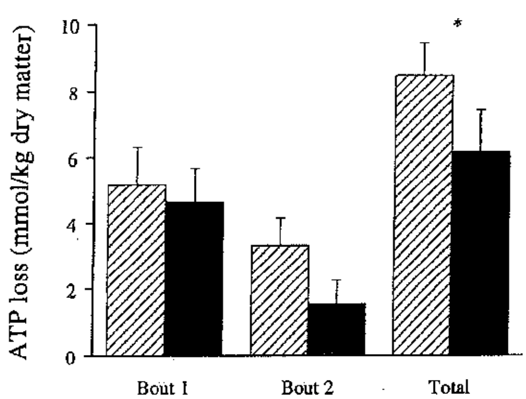

Fig. 4. Decline in mixed-muscle ATP concentration during 2 bouts of 30-s maximal-intensity isokinetic cycling exercise. Each bout of exercise was performed at 80 revolutions/min and was separated by 4 min of passive recovery. Total decline in ATP refers to sum of the decline during *bouts 1* and *2*. Values are given precreatine (hatched bars) and postcreatine (filled bars) supplementation for 5 days (4 × 5 g/day). Values represent means ± SE. *Significant differences precreatine and postcreatine supplementation ($P < 0.05$).

DISCUSSION

Dietary Cr supplementation has previously been shown to increase the rate of PCr resynthesis during recovery from maximal-intensity exercise in individuals who demonstrated close to or more than a 20 mmol/kg dry matter increase in muscle TCr concentration; in contrast, PCr resynthesis appeared to be unaffected by Cr supplementation in those individuals who demonstrated less than a 10 mmol/kg dry matter increase in muscle TCr (11). Therefore, whereas Cr supplementation evidently improved PCr resynthesis during recovery, it was clear from these results that the degree of improvement was critically dependent on the magnitude of the net accumulation of Cr in the muscle over the course of supplementation. In agreement with these findings, the results of the present experiment demonstrated that, although all subjects appeared to benefit from an improvement in exercise performance after Cr ingestion, the degree of improvement was clearly related to the magnitude of Cr accumulation in

Table 2. *PCr concentration measured in type I and type II muscle fibers immediately before and after 2 bouts of 30-s maximal-intensity isokinetic cycling exercise*

Fiber Type	Pre-Cr Ingestion				Post-Cr Ingestion			
	Pre-B1	Post-B1	Pre-B2	Post-B2	Pre-B1	Post-B1	Pre-B2	Post-B2
I	66.6 ± 4.2	18.7 ± 2.9*	62.0 ± 2.2	19.3 ± 3.5*	77.6 ± 3.2†	29.9 ± 6.0*	69.6 ± 3.9	12.8 ± 3.7*
II	79.3 ± 1.5	15.8 ± 5.0*	55.5 ± 4.2	10.8 ± 2.5*	91.0 ± 5.8†	17.9 ± 9.8*	67.2 ± 4.1	7.4 ± 2.8*

Values are means ± SE in mmol/kg dry matter. Each bout of exercise was performed at 80 revolutions/min and was separated by 4 min of recovery. Values are given precreatine and postcreatine supplementation for 5 days (4 × 5 g/day). * Significant differences pre- and postexercise ($P < 0.01$). † Significant differences between corresponding concentrations pre- and post-Cr ingestion ($P \le 0.05$).

the muscle. This association is illustrated by Fig. 3, which shows that changes in both peak work production ($r = 0.71$, $P < 0.05$; Fig. 3*A*) and total work production ($r = 0.71$, $P < 0.05$; Fig. 3*B*) were related to the change in muscle TCr concentration and that the magnitude of the increase in muscle TCr concentration required to produce improvements in exercise performance was of the same order as that previously shown to facilitate PCr resynthesis (11).

Clearly, these data point to the importance of maximizing the increase in muscle TCr concentration when attempting to increase exercise performance via Cr supplementation. Previously published results (11, 16) indicate that the muscle TCr concentration before supplementation is an important determinant of subsequent increases in muscle TCr concentration during Cr ingestion. However, the present results demonstrate that the initial TCr concentration is not the sole determinant. *Subjects 2* and *3* depicted in Fig. 2, for example, had the same initial TCr concentration; however, *subject 2*, for no obviously apparent reason, experienced a sixfold greater increase in muscle TCr concentration during supplementation. Given that animal studies have demonstrated both dietary and hormonal effects on Cr biosynthesis and muscle Cr uptake and retention (28), future work aimed at maximizing muscle Cr uptake during supplementation might focus on elucidating the principle factors regulating uptake in humans.

The present study extends previously published work that has also demonstrated that Cr supplementation can improve performance during maximal-intensity exercise (1, 5, 8, 12, 17), by performing a direct investigation of the effects of Cr supplementation on skeletal muscle energy metabolism. The 30% reduction in mixed-muscle ATP loss found during exercise in the present study, together with the observed increase in total work production, suggests that ADP rephosphorylation to ATP during exercise was improved as a consequence of Cr supplementation. This hypothesis is supported by previous studies in which an improvement in exercise performance after Cr ingestion was accompanied by a reduction in plasma ammonia (5, 12) and hypoxanthine (1) accumulation during exercise, since both of these metabolites are accepted markers of muscle adenine nucleotide loss during maximal-intensity exercise (15).

However, the factor(s) underlying the increase in exercise performance and concommitant reduction in ATP loss is not readily apparent from the mixed-muscle metabolite data of the present experiment. After Cr supplementation, the change in resting mixed-muscle PCr concentration ranged from −3.2 to 26.9 mmol/kg dry matter. Given that the rate of ATP resynthesis from PCr over the course of 30 s of maximal-intensity exercise is likely to be in the region of 1.5 mmol·kg dry matter^{-1}·s^{-1} (20), the larger increases in resting PCr concentration within this range may have made a significant contribution to total ATP production during exercise. However, no correlation was found between the change in mixed-muscle PCr concentration after Cr ingestion and subsequent changes in either mixed-muscle ATP loss or exercise performance. Somewhat unexpectedly, no correlation was found in the present study between the increase in muscle TCr concentration and mixed-muscle ATP loss, although presumably this finding is attributable to the observation that subjects with the greatest increase in muscle TCr concentration also showed the greatest increase in total

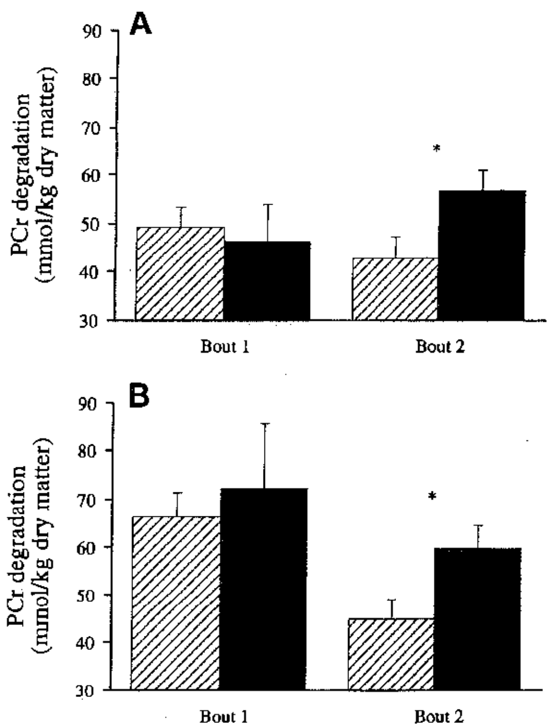

Fig. 5. Phosphocreatine (PCr) degradation in type I (*A*) and type II (*B*) fibers during 2 bouts of 30-s maximal-intensity isokinetic cycling exercise. Each bout of exercise was performed at 80 revolutions/min and was separated by 4 min of passive recovery. Values are given precreatine (hatched bars) and postcreatine (filled bars) supplementation for 5 days (4 × 5 g/day). Values represent means ± SE. *Significant differences precreatine and postcreatine supplementation ($P < 0.05$).

work production, i.e., the ATP requirement was higher, and the subsequent reduction in ATP loss observed during exercise was therefore proportionally lower.

One explanation for these findings may be that favorable metabolic effects arising from Cr supplementation were confined principally to one muscle fiber type, making the metabolic responses recorded in mixed muscle more difficult to interpret. Evidence is available showing that, although the resting PCr concentration of type II fibers is ~12% greater than that of type I fibers (13, 25, 26, 27), the rate of PCr degradation during 30 s of maximal-intensity dynamic exercise is 10–25% greater in type II muscle fibers (13, 27). Furthermore, the rate of PCr degradation was found to be 33% greater in type II compared with type I fibers during 20 s of high-intensity electrically evoked isometric contraction. During the final 10 s of contraction, a 60% fall in the rate of type II fiber PCr degradation was observed, whereas the rate of PCr degradation was reduced by 15% in type I fibers (25). Accordingly, the authors attributed the decline in muscle force during contraction to a fall in energy provision from PCr in type II fibers. It is reasonable to suggest, therefore, that Cr supplementation might exert a greater effect on this fiber type during maximal-intensity exercise. The present finding that the change in type II fiber PCr concentration before *exercise bouts 1* and *2* was positively correlated with both a change in PCr degradation during exercise in this fiber type (r = 0.78, $P < 0.01$) and with a change in total work production (r = 0.66, $P < 0.05$) suggests that the increase in type II fiber PCr availability resulting from Cr ingestion may indeed have been responsible for the improvements found in exercise performance. Further support for this suggestion is provided by the observation that the change in type I fiber PCr concentration after Cr ingestion was unrelated to changes in both type I fiber PCr degradation during exercise (r = 0.22) and total work production (r = 0.32).

These findings suggest that the increase in type II fiber PCr concentration resulting from Cr supplementation might account for the observed increase in exercise performance. However, the increase in PCr concentration is likely to reflect an overall increase in the fiber TCr concentration, which may itself be responsible for improvements in exercise performance by increasing mitochondrial ATP production in type II fibers during exercise and recovery. This suggestion is supported by studies showing that the increase in muscle TCr concentration after Cr supplementation was principally in the form of Cr (11, 16), which was in turn likely to be responsible for accelerating the rate of PCr resynthesis in human muscle during recovery from intense ischemic contraction (11). In vitro studies have demonstrated that Cr can increase the rate of respiration in skeletal muscle mitochondria (3) and skinned cardiac muscle fibers (9), and the role of Cr as an acceptor of mitochondrial ATP has been discussed in a series of papers (3, 4, 23, 29). The uptake of Cr by different muscle fiber types was not investigated in the present study. However, given that the TCr concentration of fast contracting skeletal muscle is 45% greater than that of slow muscle (10) and that the increase in PCr concentration was greater in type II fibers in the present study, it appears likely that Cr uptake was greater in this fiber type.

Peak work production during *exercise bouts 1* and *2* occurred within 2 s of the onset of exercise in the present study, and a possible improvement in peak work production as a result of an increase in energy substrate availability seems unlikely. However, both an increase in peak power output during isokinetic cycling (5) and an increase in maximal strength (8) have been reported after Cr supplementation. The mechanism(s) responsible for these improvements in performance are unknown but may be related to the increase in body mass found after Cr supplementation by Balsom et al. (1) and Greenhaff et al. (11), which may be related in turn to an increase in fat-free mass (8). Whether these changes can explain the improvements seen in maximal work production and strength needs to be investigated.

In agreement with previous studies in which blood lactate production during maximal-intensity exercise was unchanged after Cr ingestion (5, 12), mixed-muscle lactate production during exercise appeared to be unaffected by Cr ingestion in the present study. However, a decrease in blood lactate accumulation has been observed during repeated bouts of maximal-intensity exercise lasting 6 s and separated by recovery periods of 30 s duration (1). One possible explanation for this discrepancy lies with the duration of exercise; the dephosphorylation of ADP to AMP during an exercise period of 6 s has the potential to be buffered by PCr to a greater effect than during maximal-intensity exercise lasting 30 s, since the PCr store of skeletal muscle has been shown to decrease by >50% within the first 10 s of maximal-intensity exercise (19). The accumulation of free AMP, a known allosteric activator of phosphorylase *a* (22) and thereby the rate of glycogenolysis, would therefore be proportionally lower, and more likely to be buffered effectively by the increase in PCr resulting from Cr supplementation, than would be the case during maximal-intensity exercise lasting 30 s. If so, then the lower lactate production found after Cr ingestion by Balsom et al. (1) may be attributable to improved buffering of ADP dephosphorylation, and thereby AMP accumulation, during successive bouts of exercise lasting 6 s.

In conclusion, the present study has demonstrated a positive relationship between increases in muscle TCr concentration and improvements in performance during maximal dynamic exercise. The results suggest that this ergogenic effect can be attributed to increased ATP resynthesis during exercise, as a consequence of increased PCr availability in type II muscle fibers.

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EXHIBIT U

Chemical kinetics

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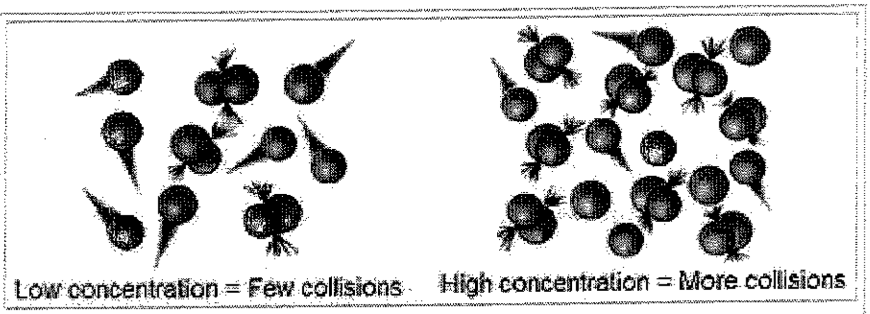


Reaction rate tends to increase with concentration - a phenomenon explained by collision theory.

Chemical kinetics, also known as **reaction kinetics**, is the study of rates of chemical processes. Chemical kinetics includes investigations of how different experimental conditions can influence the speed of a chemical reaction and yield information about the reaction's mechanism and transition states, as well as the construction of mathematical models that can describe the characteristics of a chemical reaction. In 1864, Peter Waage and Cato Guldberg pioneered the development of chemical kinetics by formulating the law of mass action, which states that the speed of a chemical reaction is proportional to the quantity of the reacting substances.

Chemical kinetics deals with the experimental determination of reaction rates from which rate laws and rate constants are derived. Relatively simple rate laws exist for zero-order reactions (for which reaction rates are independent of concentration), first-order reactions, and second-order reactions, and can be derived for others. In consecutive reactions, the rate-determining step often determines the kinetics. In consecutive first-order reactions, a steady state approximation can simplify the rate law. The activation energy for a reaction is experimentally determined through the Arrhenius equation and the Eyring equation. The main factors that influence the reaction rate include: **the physical state of the reactants, the concentrations of the reactants, the temperature at which the reaction occurs, and whether or not any catalysts are present in the reaction.**

Contents

Factors affecting reaction rate

Nature of the reactants

Depending upon what substances are reacting, the reaction rate varies. Acid/base reactions, the formation of salts, and ion exchange are fast reactions. When covalent bond formation takes place between the molecules and when large molecules are formed, the reactions tend to be very slow. Nature and strength of bonds in reactant molecules greatly influence the rate of its transformation into products.

Physical state

The physical state (solid, liquid, or gas) of a reactant is also an important factor of the rate of change. When reactants are in the same phase, as in aqueous solution, thermal motion brings them into contact. However, when they are in different phases, the reaction is limited to the interface between the reactants. Reaction can occur only at their area of contact; in the case of a liquid and a gas, at the surface of the liquid. Vigorous shaking and stirring may be needed to bring the reaction to completion. This means that the more finely divided a solid or liquid reactant the greater its surface area per unit volume and the more contact it makes with the other reactant, thus the faster the reaction. To make an analogy, for example, when one starts a fire, one uses wood chips and small branches — one does not start with large logs right away. In organic chemistry, on water reactions are the exception to the rule that homogeneous reactions take place faster than heterogeneous reactions.

Concentration

Concentration plays a very important role in reactions, because, according to the collision theory of chemical reactions, molecules must collide in order to react together. As the concentration of the reactants increases, the frequency of the molecules colliding increases, striking each other more frequently by being in closer contact at any given point in time. One may think of two reactants being in a closed container: All the molecules contained within are colliding constantly. By increasing the amount of one or more of the reactants, these collisions happen more often, increasing the reaction rate.

Temperature

Temperature usually has a major effect on the rate of a chemical reaction. Molecules at a higher temperature have more thermal energy. Although collision frequency is greater at higher temperatures, this alone contributes only a very small proportion to the increase in rate of reaction. Much more important is the fact that the proportion of reactant molecules with sufficient energy to react (energy greater than activation energy: $E > E_a$) is significantly higher and is explained in detail by the Maxwell–Boltzmann distribution of molecular energies.

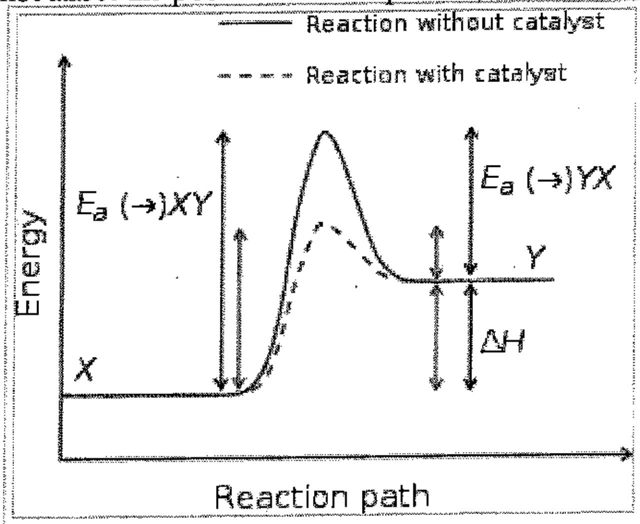
The 'rule of thumb' that the rate of chemical reactions doubles for every 10 °C temperature rise is a common misconception. This may have been generalized from the special case of biological systems, where the α (temperature coefficient) is often between 1.5 and 2.5.

A reaction's kinetics can also be studied with a temperature jump approach. This involves using a sharp rise in temperature and observing the relaxation time of the return to equilibrium.

Catalysts

A catalyst is a substance that accelerates the rate of a chemical reaction but remains chemically unchanged afterwards. The catalyst increases rate reaction by providing a different reaction mechanism to occur with a lower

activation energy. In autocatalysis a reaction product is itself a catalyst for that reaction leading to positive feedback. Proteins that act as catalysts in biochemical reactions are called enzymes. Michaelis-Menten kinetics describe the rate of enzyme mediated reactions. A catalyst does not affect the position of the equilibria, as the catalyst speeds up the backward and forward reactions equally.



Generic potential energy diagram showing the effect of a catalyst in an hypothetical endothermic chemical reaction. The presence of the catalyst opens a different reaction pathway (shown in red) with a lower activation energy. The final result and the overall thermodynamics are the same.

In certain organic molecules, specific substituents can have an influence on reaction rate in neighbouring group participation.

Agitating or mixing a solution will also accelerate the rate of a chemical reaction, as this gives the particles greater kinetic energy, increasing the number of collisions between reactants and, therefore, the possibility of successful collisions.

Pressure

Increasing the pressure in a gaseous reaction will increase the number of collisions between reactants, increasing the rate of reaction. This is because the activity of a gas is directly proportional to the partial pressure of the gas. This is similar to the effect of increasing the concentration of a solution.

A reaction's kinetics can also be studied with a pressure jump approach. This involves making fast changes in pressure and observing the relaxation time of the return to equilibrium.

SURFACE AREA OF SOLID REACTANT: On increasing the surface area, rate of reaction will also increase.

Equilibrium

While chemical kinetics is concerned with the rate of a chemical reaction, thermodynamics determines the extent to which reactions occur. In a reversible reaction, chemical equilibrium is reached when the rates of the forward and reverse reactions are equal and the concentrations of the reactants and products no longer change. This is demonstrated by, for example, the Haber–Bosch process for combining nitrogen and hydrogen to produce ammonia. Chemical clock reactions such as the Belousov–Zhabotinsky reaction demonstrate that component concentrations can oscillate for a long time before finally attaining the equilibrium.

Free energy

In general terms, the free energy change (ΔG) of a reaction determines whether a chemical change will take place, but kinetics describes how fast the reaction is. A reaction can be very exothermic and have a very positive entropy change but will not happen in practice if the reaction is too slow. If a reactant can produce two different products, the thermodynamically most stable one will in general form, except in special circumstances when the reaction is said to be under kinetic reaction control. The Curtin–Hammett principle applies when determining the product ratio for two reactants interconverting rapidly, each going to a different product. It is possible to make predictions about reaction rate constants for a reaction from free-energy relationships.

The kinetic isotope effect is the difference in the rate of a chemical reaction when an atom in one of the reactants is

replaced by one of its isotopes.

Chemical kinetics provides information on residence time and heat transfer in a chemical reactor in chemical engineering and the molar mass distribution in polymer chemistry.

Applications

The mathematical models that describe chemical reaction kinetics provide chemists and chemical engineers with tools to better understand and describe chemical processes such as food decomposition, microorganism growth, stratospheric ozone decomposition, and the complex chemistry of biological systems. These models can also be used in the design or modification of chemical reactors to optimize product yield, more efficiently separate products, and eliminate environmentally harmful by-products. When performing catalytic cracking of heavy hydrocarbons into gasoline and light gas, for example, kinetic models can be used to find the temperature and pressure at which the highest yield of heavy hydrocarbons into gasoline will occur. Kinetics is also a basic aspect of chemistry.

See also

- Collision theory
- Arrhenius equation
- Autocatalytic reactions and order creation
- Flame speed
- Detonation
- Two-dimensional gas
- Enthalpy
- Reaction progress kinetic analysis

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- Washington state university (http://www.sci.wsu.edu/idea/ChemKinetics/)
- Chemical Kinetics of Gas Phase Reactions (http://mark.jelezniak.de/Chemked/index.htm)
- Chemical Kinetics Summary (http://www.shodor.org/UNChem/advanced/kin/index.html)
- Kinpy: Python code generator for solving kinetic equations (http://code.google.com/p/kinpy/)
- PottersWheel Matlab toolbox to fit chemical rate constants to experimental data

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Collision theory

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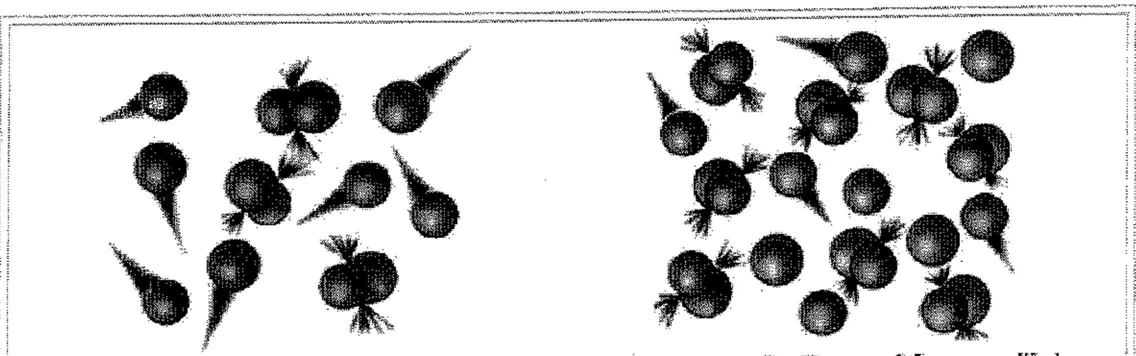

Reaction rate tends to increase with concentration phenomenon explained by **collision theory**

Collision theory is a theory proposed by [1] William Lewis in 1916 and 1918, that qualitatively explains how chemical reactions occur and why reaction rates differ for different reactions.[2] The collision theory can only occur when the suitable particles of the reactant hit with each other. Only a certain percentage of the sum of the collisions cause any noticeable or significant chemical change; these successful changes are called successful collisions. The successful collisions have enough energy, also known as activation energy, at the moment of impact to break the preexisting bonds and form all new bonds. This results in the products of the reaction. Increasing the concentration of the reactant particles and raising the temperature, thus bringing about more collisions and therefore much more successful collisions, which increases the rate of reaction.

When a catalyst is involved in the collision between the reactant molecules, less energy is required for the chemical change to take place, and hence more collisions have sufficient energy for reaction to occur. The reaction rate therefore increases.

Collision theory is closely related to chemical kinetics.

Contents

Rate constant

The rate constant for a bimolecular gas phase reaction, as predicted by collision theory is:

$$k(T) = Z\rho\exp\left(\frac{-E_a}{RT}\right).$$

where:

- Z is the collision frequency.[3]
- ρ is the steric factor. [4]
- E_a is the activation energy of the reaction.
- T is the temperature.
- R is gas constant.

The collision frequency is:

$$Z = N_A\sigma_{AB}\sqrt{\frac{8k_BT}{\pi\mu_{AB}}}$$

where:

- N_A is the Avogadro constant
- σ_{AB} is the reaction cross section
- k_B is Boltzmann'''s constant
- μ_{AB} is the reduced mass of the reactants.

Quantitative insights

Derivation

Consider the reaction:

$$A + B \rightarrow C$$

In collision theory it is considered that two particles A and B will collide if their nuclei get closer than a certain distance. The area around a molecule A in which it can collide with an approaching B molecule is called the cross section (σ_{AB}) of the reaction and is, in principle, the area corresponding to a circle whose radius (r_{AB}) is the sum of the radii of both reacting molecules, which are supposed to be spherical. A moving molecule will therefore sweep a volume $\pi r_{AB}^2 c_A$ per second as it moves, where c_A is the average velocity of the particle.

From kinetic theory it is known that a molecule of A has an average velocity (different from root mean square velocity) of $c_A = \sqrt{\frac{8k_BT}{\pi m_A}}$, where k_B is Boltzmann constant and m_A is the mass of the molecule.

The solution of the two body problem states that two different moving bodies can be treated as one body which has the reduced mass of both and moves with the velocity of the center of mass, so, in this system μ_{AB} must be used instead of m_A.

Therefore, the total **collision frequency**,[3] of all A molecules, with all B molecules, is:

$$N_A^2\sigma_{AB}\sqrt{\frac{8k_BT}{\pi\mu_{AB}}}[A][B] = N_A^2 r_{AB}^2\sqrt{\frac{8\pi k_BT}{\mu_{AB}}}[A][B] = Z[A][B]$$

From Maxwell Boltzmann distribution it can be deduced that the fraction of collisions with more energy than the activation energy is $e^{\frac{-E_a}{k_BT}}$. Therefore the rate of a bimolecular reaction for ideal gases will be:

$$r = Z\rho[A][B]\exp\left(\frac{-E_a}{RT}\right)$$

Where:

- Z is the collision frequency.
- ρ is the steric factor, which will be discussed in detail in the next section.
- E_a is the activation energy of the reaction.
- T is the absolute temperature.
- R is gas constant.

The product $Z\rho$ is equivalent to the preexponential factor of the Arrhenius equation.

Validity of the theory and steric factor

Once a theory is formulated, its validity must be tested, that is, compare its predictions with the results of the experiments.

When the expression form of the rate constant is compared with the rate equation for an elementary bimolecular reaction, $r = k(T)[A][B]$, it is noticed that $k(T) = N_A^2\sigma_{AB}\sqrt{\frac{8k_BT}{\pi m_A}}\exp\left(\frac{-E_a}{RT}\right)$.

That expression is similar to the Arrhenius equation, and gives the first theoretical explanation for the Arrhenius equation on a molecular basis. The weak temperature dependence of the preexponential factor is so small compared to the exponential factor that it cannot be measured experimentally, that is, *"it is not feasible to establish, on the basis of temperature studies of the rate constant, whether the predicted $T^{1/2}$ dependence of the preexponential factor is observed experimentally"*[citation needed]

Steric factor

If the values of the predicted rate constants are compared with the values of known rate constants it is noticed that collision theory fails to estimate the constants correctly and the more complex the molecules are, the more it fails. The reason for this is that particles have been supposed to be spherical and able to react in all directions; that is not true, as the orientation of the collisions is not always the right one. For example in the hydrogenation reaction of ethylene the H_2 molecule must approach the bonding zone between the atoms, and only a few of all the possible collisions fulfill this requirement.

To alleviate this problem, a new concept must be introduced: the **steric factor**, ρ. It is defined as the ratio between the experimental value and the predicted one (or the ratio between the frequency factor and the collision frequency, and it is most often less than unity.[4]

$$\rho = \frac{A_{observed}}{Z_{calculated}}$$

Usually, the more complex the reactant molecules, the lower the steric factor. Nevertheless, some reactions exhibit steric factors greater than unity: the harpoon reactions, which involve atoms that exchange electrons, producing ions. The deviation from unity can have different causes: the molecules are not spherical, so different geometries are possible; not all the kinetic energy is delivered into the right spot; the presence of a solvent (when applied to solutions), etc.

Experimental rate constants compared to the ones predicted by collision theory for gas phase reactions

Reaction	A (Azra frequency factor)	Z (collision frequency)	Steric factor
$2ClNO \rightarrow 2Cl + 2NO$	$9.4\ 10^{9}$	$5.9\ 10^{10}$	0.16
$2ClO \rightarrow Cl_2 + O_2$	$6.3\ 10^{7}$	$2.5\ 10^{10}$	$2.3\ 10^{-3}$
$H_2 + C_2H_4 \rightarrow C_2H_6$	$1.24\ 10^{6}$	$7.3\ 10^{11}$	$1.7\ 10^{-6}$
$Br_2 + K \rightarrow KBr + Br$	10^{12}	$2.1\ 10^{11}$	4.3

Collision theory can be applied to reactions in solution; in that case, the *solvent cage* has an effect on the reactant molecules and several collisions can take place in a single encounter, which leads to predicted preexponential factors being too large. ρ values greater than unity can be attributed to favorable entropic contributions.

Experimental rate constants compared to the ones predicted by collision theory for reactions in solution[5]

Reaction	Solvent	$A\ 10^{-11}$	$Z\ 10^{-11}$	Steric factor
$C_2H_5Br + OH^-$	C_2H_5OH	4.30	3.86	1.11
$C_2H_5O^- + CH_3I$	C_2H_5OH	2.42	1.93	1.25
$ClCH_2CO_2^- + OH^-$	water	4.55	2.86	1.59
$C_3H_6Br_2 + I^-$	CH_3OH	1.07	1.39	0.77
$HOCH_2CH_2Cl + OH^-$	water	25.5	2.78	9.17
$4\text{-}CH_3C_6H_4O^- + CH_3I$	ethanol	8.49	1.99	4.27
$CH_3(CH_2)_2Cl + I^-$	$(CH_3)_2CO$	0.085	1.57	0.054
$C_5H_5N + CH_3I$	$C_2H_2Cl_4$	-	-	$2.0\ 10^{-6}$

See also

- Two-dimensional gas

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- Introduction to Collision Theory (http://www.chemguide.co.uk/physical/basicrates/introduction.html)

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EXHIBIT V

6

Insulin action on protein metabolism

GIANNI BIOLO
ROBERT R. WOLFE

Insulin is probably the most important regulator of protein metabolism, but many aspects of its action in vivo are not fully understood. In most circumstances insulin is anabolic, meaning that it stimulates net protein synthesis. Thus, patients with uncontrolled type I diabetes, due to lack of insulin, tend to have muscle wasting that is rapidly reversed during insulin treatment (Kimball et al, 1989). Moreover, hyperinsulinaemia in normal subjects acutely decreases plasma concentrations of essential amino acids as a consequence of net protein deposition (Fukagawa et al, 1986; Tessari et al, 1986b). While the overall anabolic effect of insulin on protein metabolism is well established, uncertainties remain about the mechanisms and the sites of its action.

An anabolic hormone can potentially exert its effect by stimulating protein synthesis and/or inhibiting protein breakdown. Furthermore, the process of synthesis and breakdown can be directly affected by the rate of transmembrane transport of amino acids, which in turn may be under hormonal control. We will therefore consider the role of insulin in the control of protein synthesis, breakdown and amino acid transport. Focus will be primarily on the in vivo studies.

BIOCHEMICAL BASIS OF PROTEIN TURNOVER AND AMINO ACID TRANSPORT

Protein breakdown

The hydrolysis of intracellular proteins to their constituent amino acids is a highly regulated process that recent studies have revealed to be far more complex than previously believed (Kettelhut et al, 1988). Three major pathways have been identified. The most thoroughly characterized is an ATP-independent system of acid proteases (cathepsins) and hydrolases contained in cellular organelles termed lysosomes (De Duve, 1969). The other two pathways are the Ca^{2+}-dependent proteases (Mellgren, 1987) and an ATP-dependent pathway requiring the presence of ubiquitin (Fagan et al, 1987). Insulin apparently plays an important role only in the regulation of lysosome protein breakdown (Jefferson et al, 1974; Mortimore et al, 1978;

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Kettelhut et al, 1988). Under normal physiological conditions, lysosomes are predominantly involved in the degradation of extracellular and membrane-associated proteins in liver (Furuno and Goldberg, 1986; Kettelhut et al, 1988). Muscle lysosomes do not normally play an important role in myofibrillar protein degradation (Lowell et al, 1986). However, during insulin withdrawal lysosomes are involved in muscle protein breakdown (Kettelhut et al, 1988). The other proteases are located in the cytosol and show proteolytic activity at neutral pH. The Ca^{2+}-dependent proteases are unresponsive to insulin (Kettelhut et al, 1988) and need unphysiologically elevated Ca^{2+} concentrations, and therefore they may be important primarily in damaged tissues (Furuno and Goldberg, 1986). The most important process in muscle is the ATP-independent system that requires the presence of a specialized protein termed ubiquitin (Fagan et al, 1987). This system is not sensitive to insulin (Kettelhut et al, 1988) and is quantitatively the most important degradative system of myofibrillar proteins in muscle under normal conditions (Furuno and Goldberg, 1986). From these in vitro findings, it would be predicted that in vivo insulin lack could result in accelerated breakdown of both liver and muscle protein, and insulin excess would be expected to diminish protein breakdown primarily in the liver.

Protein synthesis

The first step in protein synthesis occurs in the nucleus of the cells and involves the transcription of a specific gene into messenger RNA (mRNA) molecules. mRNAs are transported into the cytosol, where they associate with ribosomes for the translation of the base sequence into an amino acid sequence. The translation process can be divided in three phases: initiation, elongation and termination of the peptide chain. Many ribosomes can associate with mRNA molecules, forming polyribosomes. A polyribosome can synthesize several peptide chains from a single mRNA. Cytoplasmic free amino acids are not directly utilized for protein synthesis. The translation process involves the binding of amino acids to specific transfer RNA (tRNA) molecules, forming aminoacyl-tRNAs. The aminoacyl-tRNAs constitute very small pools that are turning over rapidly. Insulin has been shown to stimulate both the transcription and translation processes for specific proteins (Kimball and Jefferson, 1988). Gene expression (as reflected by mRNA levels) for many proteins has been shown to be stimulated by insulin, including albumin and myosin heavy chain α (Dillman, 1988) in liver and skeletal muscle, respectively. The mRNA content reflects the ultimate potential for protein synthesis in a tissue, but may not be directly related to the rate of translation and thus synthesis of protein (Wolfe, 1992). Insulin also enhances the translation process in skeletal muscle by stimulating peptide chain initiation (Jefferson et al, 1974; Fulks and Goldberg, 1975). Thus, from the molecular basis it would be expected that in vivo insulin would generally increase the overall capacity for protein synthesis (mRNA content) and stimulate the translation and production of specific proteins, such as muscle proteins.

Transmembrane amino acid transport

Intracellular free amino acids exchange with the extracellular pool through a complex system of active transporters located on the plasma membrane. There are several different carriers, which have overlapping specificity for the different amino acids (Christensen, 1990). The process involves active transport (Christensen, 1990), which enables the intracellular concentration to be higher than the plasma concentration (Bergstrom et al, 1974). However, the plasma–intracellular gradient is dramatically variable among different amino acids (Bergstrom et al, 1974), suggesting different kinetic characteristics of the various systems (Biolo et al, 1992). A stimulatory effect of insulin on amino acid uptake has been reported in a variety of tissues studied in vitro (Shotwell et al, 1983), including skeletal muscle, heart and liver. However, insulin predominantly affects the activity of transport system A (Shotwell et al, 1983). System A transports amino acids with short polar side-chains such as alanine and glycine. A glutamine transporting system in skeletal muscle, termed N^n, was also found to be responsive to insulin (Hundal et al, 1987). Transmembrane amino acid transport systems are potentially important regulators of protein metabolism, because they can regulate intracellular amino acid availability for protein synthesis. The in vivo importance of alterations in amino acid transport in regulating protein synthesis can be ascertained only by quantification of transport rates in relation to the rate of appearance of amino acids intracellularly from the process of protein breakdown. We have recently described a new tracer method that allows dynamic quantitation of these rates (Biolo et al, 1992). We have found in dogs that 20–50% of the intracellular pool comes from plasma, depending on the specific amino acid. From this observation, it might be expected that regulation of plasma uptake would be important in determining availability of intracellular amino acids for protein synthesis, because as much as half of the intracellular pool comes from the plasma. However, there is no experimental evidence that insulin affects amino acid transport in an important way, as only the transport of alanine, and possibly of glutamine (non-essential amino acids produced readily inside the muscle cells), is stimulated in vitro by insulin. There are no data from in vivo experiments on this potential site of action of insulin.

PHYSIOLOGICAL EFFECTS OF INSULIN AT WHOLE BODY LEVEL

The overall anabolic effect of insulin at the whole body level is well established. Early studies have shown that in insulin-deprived type I diabetics nitrogen balance is markedly negative, indicating a net catabolism of body protein (Atchley et al, 1933). More recently, leucine turnover studies have suggested that during insulin deficiency the net catabolic state arises from an increase in whole body protein breakdown (Nair et al, 1983, 1987; Rogert et al, 1985; Tessari et al, 1986a; Umpleby et al, 1986; Pacy et al, 1989). Leucine oxidation was also found to be increased with insulin

deficiency, indicating an increased net loss of an essential amino acid (Nair et al, 1983, 1987; Rogert et al, 1985; Tessari et al, 1986a; Umpleby et al, 1986; Pacy et al, 1989). Paradoxically, in the same studies, non-oxidative leucine disposal, an index of whole body protein synthesis, was found to be normal or increased in insulin-deprived diabetics. The unexpected result of unimpaired protein synthesis during insulin deficiency may represent a methodological artefact, as the value is not measured directly but deduced mathematically. However, if insulin deficiency does cause an increase in the rate of whole body protein synthesis, it raises the question of where this increased synthesis is occurring. Some liver secretory proteins involved in the acute-phase response are increased during insulin deficiency (McMillan, 1970; Jonsson and Wales, 1976). Gut and kidney protein content was also found to be increased in diabetic rats (McNurlan and Garlic, 1981; Kimball et al, 1989), indicating a stimulation of synthesis with insulin lack. More expectedly, in diabetes muscle protein synthesis was found to be normal (Pacy et al, 1989) or decreased (Pain and Garlic, 1974; Pain et al, 1983).

The apparent discrepancy between whole body responses to insulin and the predictions, from in vitro studies, that insulin should stimulate synthesis can be partially explained by the apparent potent effect of insulin to whole protein breakdown in vivo. Using the hyperinsulinaemic euglycaemic clamp technique combined with leucine turnover, insulin has been found to reduce whole body protein breakdown in a dose-dependent manner (Fukagawa et al, 1985; Tessari et al, 1986b; Shangraw et al, 1988). At physiological insulin concentrations, protein breakdown is suppressed up to 20–30% (Fukagawa et al, 1985; Tessari et al, 1986b, 1987; Castellino et al, 1987; Shangraw et al, 1988). Leucine oxidation is also blunted by insulin (Fukagawa et al, 1985; Tessari et al, 1986b, 1987; Castellino et al, 1987; Shangraw et al, 1988). Thus, by inhibiting protein breakdown, insulin has the effect of reducing the availability of precursor for synthesis of new proteins. Infusion of amino acids without insulin stimulates whole body protein synthesis (Castellino et al, 1987; Tessari et al, 1987). When amino acids are infused during euglycaemic hyperinsulinaemia, insulin does not affect protein synthesis (Castellino et al, 1987; Tessari et al, 1987). Thus, whereas these data indicate that insulin does not inhibit protein synthesis directly, there are no data at the whole body level to support the role of insulin as a stimulator of protein synthesis.

Thus, there is a considerable discrepancy between the in vivo studies, indicating no direct effect of insulin on protein synthesis, and the in vitro experiments showing an important stimulatory effect of insulin on specific proteins. Furthermore, although only certain specific proteins have been shown to be stimulated by insulin in vitro, these include the quantitatively most important contributors to whole body protein turnover (muscle protein, collagen and albumin) (Dillmann, 1988; Goldstain et al, 1989). Thus, if this action was occurring in vivo it would be expected that this would be reflected by the whole body technique.

The best explanation for this discrepancy may be that limitations of the whole body technique make quantitative assessment of protein synthesis unreliable. In the leucine technique, the rate of protein synthesis is

calculated as the difference between protein breakdown and leucine oxidation. Neither breakdown nor oxidation is determined precisely (Wolfe, 1992), for a number of reasons. The most important problem in relation to insulin is probably that each tissue is assumed to contribute to the observed plasma enrichment of leucine (or α-ketoisocaproate) in proportion to the rate of protein breakdown occurring in that tissue, and yet owing to considerable intertissue differences in both breakdown and transport rates, this assumption is not valid. As insulin has different effects in individual tissues, it is reasonable to assume that the pooling that necessarily occurs with the whole body method might lead to erroneous conclusions, particularly when it is considered that the rate of synthesis is a derived value from the difference between two imprecise terms. The different effect of insulin on tissues is shown, for example, by the fact that plasma amino acid concentration is markedly reduced by insulin, and yet muscle plays no role in this response (Tessari et al, 1990, 1991). On the other hand, from the data presented above, we cannot exclude the possibility that in vivo insulin has no effect on protein synthesis, or has a sufficiently inhibitory effect on some proteins to counterbalance its stimulatory effect on other proteins. Thus, to understand the in vivo role of insulin, it is necessary to examine responses at the tissue level.

METHODS FOR STUDYING TISSUE RESPONSE IN VIVO

The principal approaches for studying tissue-specific responses of protein metabolism are the measurement of fractional synthetic rate (FSR) by the direct incorporation technique, or the measurement of amino acid and protein balance by the arteriovenous (A–V) balance technique.

Measurement of protein synthesis by precursor incorporation techniques

The direct incorporation method is accomplished by either the constant infusion of tracer (Waterlow et al, 1978) or the injection of a flooding dose of tracer and tracee (Hewnshaw et al, 1971; Garlic et al, 1989). Both approaches enable determination of the rate of incorporation of the tracer into a specific protein, and then of its FSR (hours^{-1}). This refers to the rate of synthesis (e.g. g/h) divided by the total protein pool size (g). During a constant labelled amino acid infusion, it is assumed that, after equilibration, precursor enrichment is constant, so that the tracer is incorporated into the protein in a liner manner. In this case, FSR can be calculated by the following equation:

$$\mathrm{FSR} = (\mathrm{E}_{t_1} - \mathrm{E}_{t_0}) \cdot 100/[\mathrm{E}_p \cdot (t_1 - t_0)]$$

Where E_t is the enrichment (tracer/tracee) of the specific tracer amino acid in the protein at different times and E_p is the average precursor enrichment during the time period corresponding to the determination of enrichment in protein. The calculation of FSR by the short-term constant infusion technique has many practical advantages, but is limited by the need to identify

the true value of precursor enrichment (E_P). During a constant labelled amino acid infusion, not only is intracellular enrichment lower than plasma enrichment, but it is also uncertain that intracellular enrichment is actually the precursor for synthesis. In fact, most studies that have determined the enrichment of charged tRNA in a tissue (which presumably reflects the true precursor enrichment) have found the value to lie somewhere between the intracellular and extracellular free amino acid enrichment (Davey and Manchester, 1969; Martin et al, 1977; McKee et al, 1978; Everett et al, 1981).

The so-called flooding dose technique was designed to overcome the difficulty in identifying the true precursor enrichment for the calculation of protein synthesis. The rationale of the method is simultaneously to inject boluses of both tracer and tracee amino acid, thereby 'flooding' the entire precursor pool (extracellular and intracellular components) to such an extent that the enrichment is the same everywhere. The rate of synthesis is then determined by dividing the amount of tracer incorporated in a given amount of time by the average precursor enrichment over that time. There are several practical advantages to the flooding dose technique. Theoretically, it should eliminate uncertainty regarding precursor enrichment, since the precursor is at the same enrichment in all compartments. Furthermore, because of this flooding of the precursor pool, it should be possible to use only plasma enrichment measurements to quantify the true precursor enrichment. Another advantage is that it is possible to give enough tracer over time to enable sufficient tracer incorporation for accurate measurement in a short period of time. On the other hand, there are potential problems stemming from requisite assumptions. The most crucial assumption is that the bolus injection of an amount of an amino acid well in excess of the total body free pools of that amino acid will have no effect on the rate of synthesis. An additional assumption of less importance is that there is no delay in the incorporation of tracer from the free pool to the protein-bound pool, i.e. that the process of protein synthesis is instantaneous. Recent papers have addressed this technique in more detail (Chinkes et al, 1993; Toffolo et al, 1993). It appears that, at least in some circumstances, the true value is overestimated.

Arteriovenous balance technique to measure breakdown and synthesis

Measurement of tracer kinetics across a tissue have been used for many years in attempts to gain more information about regional metabolism than is available from net substrate balance alone. A–V balance of essential amino acids across a limb in combination with tracer dilution technique has been widely used to measure protein synthesis and breakdown in muscle tissue (Barret et al, 1987; Gelfand and Barret, 1987). Muscle accounts for approximately 60–70% of the volume of the limbs, and should account for the majority of limb protein turnover. This is because other limb tissues cannot contribute to a large extent to protein metabolism because of their slow turnover (bone), small protein content (fat) or small volume (skin). Few labelled amino acids have been used to trace muscle protein kinetics across a limb. In order to reflect muscle protein metabolism accurately, an

appropriate tracer amino acid should be essential, i.e. produced only from protein breakdown, and not oxidized in muscle, i.e. utilized only for protein synthesis. Phenylalanine is the most commonly used tracer. It satisfies the above requirements and has the additional advantage of equilibrating rapidly, and there is a small difference in enrichment between the intracellular and extracellular pools, reflecting the rapid transmembrane transport and the small intracellular pool of this amino acid (Biolo et al, 1992).

The measurement of phenylalanine net balance (NB) and tracer uptake across a limb requires the calculation of limb blood flow (BF) and amino acid concentration and isotope enrichment in the artery (C_A, E_A) and vein (C_V, E_V) draining the limb (i.e. femoral vein or a deep forearm vein). Then:

$$NB = (C_A - C_V) \cdot BF$$

$$\text{Tracer uptake} = (C_A \cdot E_A - C_V \cdot E_V) \cdot BF$$

Since phenylalanine is an essential amino acid not oxidized in muscle, its NB across a limb represents either the difference between amino acid influx and efflux into and from the limb, and between incorporation into and release from protein, thus reflecting the actual muscle protein balance. On the other hand, tracer uptake represents the total amount of labelled phenylalanine incorporated into protein, since tracer release from proteins is an extremely unlikely event. Like the precursor incorporation technique, accurate determination of protein synthesis with the A–V balance technique requires knowledge of the phenylalanine enrichment at the precursor pool for protein synthesis (E_p). The correct estimation of protein synthesis (PS) is obtained as follows:

$$PS = \text{Tracer uptake}/E_p$$

Since determination of phenylalanine–tRNA enrichment in muscle cannot be a routine procedure in an in vivo study, especially in humans, sampling has generally been limited to arterial and venous blood, where the enrichment of the precursor pool is systematically overestimated. The traditional A–V approach (Barret et al, 1987; Gelfand and Barret, 1987) estimates protein synthesis, quantifying the incorporation of plasma phenylalanine into muscle (R_d), as follows:

$$R_d = \text{Tracer uptake}/E_A \text{ (or in alternative: } R_d = \text{Tracer uptake}/E_V)$$

where E_A and E_V are phenylalanine enrichment in artery and vein respectively. This approach necessarily underestimates the actual value of protein synthesis (PS), by the amount of synthesis occurring from the intracellular pool derived from muscle protein breakdown (PB). Estimation of protein breakdown (R_a) is then derived by subtracting the net balance from the underestimated value of protein synthesis (R_d). Since the net balance reflects the true difference between synthesis and breakdown, the difference between synthesis and net balance will be an underestimation of breakdown. The extent of this underestimation will depend on the relative rates of synthesis and breakdown, i.e. net balance. Thus, the higher the rate of synthesis relative to breakdown, the greater the underestimation of

breakdown. The relationship between the relative underestimation of protein synthesis (R_d/PS) and breakdown (R_a/PB) and the actual rates of protein synthesis and breakdown (PS and PB respectively) can be readily derived from the following equation:

$$R_d - R_a = PS - PB$$

$$R_a = R_d - PS + PB$$

$$(R_a/PB) \cdot PB = (R_d/PS) \cdot PS - PS + PB$$

$$(R_a/PB) \cdot PB = [(R_d/PS) - 1] \cdot PS + PB$$

$$(R_a/PB) = [(R_d/PS) - 1] \cdot (PS/PB) + 1$$

From the last equation it is clear that only when PS/PB = 1, i.e. when protein synthesis equals protein breakdown, is the extent of underestimation of R_a/PB and R_d/PS equal. If PS/PB < 1, meaning negative net balance, then $R_a/PB > R_d/PS$, meaning that the breakdown rate is overestimated relative to synthesis. On the other hand, if PS/PB > 1, meaning positive net balance, then $R_a/PB < R_d/PS$, meaning that the breakdown rate is underestimated relative to synthesis. As is discussed below, insulin or amino acid infusion causes $R_a/PB < R_d/PS$, meaning that breakdown will be underestimated, relative to synthesis, by the traditional approach.

On the basis of preceding observations, it appears that traditional A–V balance model does not allow an accurate estimation of muscle protein kinetics. In particular it could lead to qualitatively erroneous conclusions with regard to regulation of protein breakdown by factors promoting muscle anabolism such as insulin and amino acids. In fact, in the case of a change in the state of muscle metabolism from catabolism (PS/PB < 1) to anabolism (PS/PB > 1) caused by an isolated increase of synthesis, the traditional A–V balance approach will erroneously reflect an apparent concomitant decrease in breakdown, since breakdown is underestimated to a lesser extent during a catabolic state than during anabolism.

An alternative approach to the A–V balance technique involves the sampling of muscle by biopsy from the limb and the measurement of amino acid enrichment in the tissue mixed free pool (E_m) (Biolo et al, 1992). Then, protein synthesis is calculated as: tracer uptake/E_M. As with the incorporation technique using a constant tracer infusion, this approach requires an assumption regarding the precursor enrichment. Thus, estimation of protein synthesis using the tissue free amino acid enrichment represents the highest possible synthetic rate, since the true precursor enrichment will not be lower than in the intracellular pool (Davey and Manchester, 1969; Martin et al, 1977; McKee et al, 1978; Everett et al, 1981). Recently, L-[1-^{13}C]leucyl-tRNA enrichment was directly measured in vivo in pig muscle during continuous L-[1-^{13}C]leucine infusion (Watt et al, 1992). From this study it was clear that the labelling of muscle leucyl-tRNA was substantially lower than that of the arterial plasma labelling of leucine and was close to that of the intracellular free leucine either in basal condition and during amino acid infusion, supporting the validity of using E_M as the precursor for protein synthesis.

PHYSIOLOGICAL EFFECTS OF INSULIN ON MUSCLE TISSUE

Skeletal muscle is the largest tissue in the body and contains 50% of body protein. Despite a slow fractional protein turnover rate, it accounts for approximately one third of total body turnover. Moreover, the catabolic effect of insulin deficiency involves primarily skeletal muscle, with visceral proteins tending to be spared (McNurlan and Garlic, 1981; Kimball et al, 1989). For these reasons the effect of insulin on muscle protein metabolism has been widely investigated both in vitro and in vivo. However, from the currently published data it is not possible to draw an unequivocal conclusion regarding the role of insulin on the regulation of muscle protein synthesis and breakdown.

A number of investigators have used the leg or forearm A–V balance technique to study, in vivo, insulin effects on human skeletal muscle. These results are summarized in Table 1. Because of the important effect of insulin in reducing amino acid concentration, the prevailing concentration of leucine (as representative of all essential amino acid concentrations) is also expressed. The variation in insulin and amino acid concentrations shown in Table 1 stems from the infusion of either or both in different experiments. Taken together, these studies suggest that the in vivo effect of insulin on net muscle protein deposition is dependent on systemic amino acid concentrations. Thus, systemic physiological hyperinsulinaemia, without amino acid infusion, failed to improve protein balance and did not modify protein synthesis or degradation, either in the forearm and the leg (Arfvidsson et al, 1991; Tessari et al, 1991). Despite the absence of an effect on muscle, in the same studies systemic amino acid concentrations and whole body protein breakdown were reduced following insulin infusion. Insulin caused these responses by acting primarily at sites other than skeletal muscle. Therefore, it is possible that the lack of a major insulin effect on muscle in these studies was related to the development of hypoaminoacidaemia.

In other studies, insulin was infused directly into the brachial artery to raise forearm deep venous insulin concentration to high physiological levels, without affecting systemic amino acid concentration, and therefore without altering amino acid delivery to the forearm. With this experimental model net protein balance increased markedly (Pozefsky et al, 1969; Gelfand and Barret, 1987), apparently owing to an isolated decrease in breakdown (Gelfand and Barret, 1987). Consistent with the interpretation of the importance of amino acid concentrations in influencing the response to insulin, systemic insulin infusion with concomitant amino acid infusion to prevent changes in systemic amino acid concentrations has been shown to improve net muscle protein balance, by means of an isolated decrease in breakdown (Heslin et al, 1992). Furthermore, hyperaminoacidaemia of two or three times the normal postabsorptive concentrations stimulates muscle protein synthesis (Gelfand et al, 1988), and such an effect is enhanced by concomitant insulin infusion (Bennet et al, 1990a). Thus, insulin at physiological postprandial concentrations promotes muscle protein anabolism only in the presence of either normal or high systemic amino acid concentrations. On the other hand, muscle protein balance is improved despite the fall

Table 1. Effect of insulin concentrations and amino acid availability on muscle protein metabolism in humans.

Reference	Insulin concentration (μU/ml)	Leucine concentration (μmol/l)	Leg or forearm	Insulin infusion	Amino acid infusion	Net protein balance	Protein synthesis	Protein breakdown
Pozefsky et al (1969)	20	118	Forearm	Local	No	↔		
Pozefsky et al (1969)	157	118	Forearm	Local	No	↑		
Gelfand and Barret (1987)	120	108	Forearm	Local	No	↑	↔	↓
Gelfand et al (1988)	14	321	Leg	No	Yes	↑	↑	↓
Bennet et al (1990a)*	100	274	Leg	Systemic	Yes	↑	↑	↓ or ↔
Tessari et al (1991)	75	95	Forearm	Systemic	No	↔	↔	↔
Arfvidsson et al (1991)	110	?	Leg	Systemic	No	↔	↔	↔
Denne et al (1991)	2614	59	Leg	Systemic	No	↑	↔	↓
Heslin et al (1992)	71	111	Forearm	Systemic	Yes	↑	↔	↓

* Differences in protein kinetics are from a comparison of amino acids plus insulin infusion versus amino acids alone.

in amino acid concentrations, at pharmacologically high insulin concentrations (Denne et al, 1991).

These recent studies established in vivo the overall response of muscle proteins to insulin infusion. However, in contrast to in vitro and animal studies, the mechanism of insulin-mediated anabolism, has generally been found to be an isolated decrease in breakdown, with no modification of synthesis. Insulin has been shown to increase muscle protein synthesis in normal volunteers only when the plasma amino acid concentration is high.

The traditional A–V balance technique allows the accurate measurement of net protein balance, but, as previously pointed out, protein synthesis and breakdown are systematically underestimated by an unpredictable and variable extent. In particular, it was demonstrated above that determination of protein breakdown with the traditional model results in a variable underestimation of the true value dependent on the net balance. Therefore, the conclusion that insulin promotes muscle anabolism directly by means of an isolated decrease in protein breakdown without an effect on protein synthesis may not be conclusive.

In contrast to the findings in humans with the A–V tracer balance technique, Pain and colleagues used both the constant infusion (Pain and Garlic, 1974) and the flooding dose (Pain et al, 1983) techniques in intact rats and found that the fractional rate of protein synthesis was decreased in the muscle of diabetic rats and was improved by insulin treatment. Also, in normal rats, insulin infusion increased muscle protein synthesis and this response was enhanced by concomitant hyperaminoacidaemia (Garlic and Grant, 1988).

It is difficult to find a physiological explanation to account for the discrepancies between in vitro and in vivo findings and between animal and human data. These contradictions probably stem from the complex problem of determining protein synthesis and breakdown in vivo. Therefore, further investigations are required, using more accurate techniques, to understand the mechanism of the muscle anabolic response to insulin in humans.

PHYSIOLOGICAL EFFECTS OF INSULIN ON OTHER TISSUES

The effect of insulin on protein metabolism in the liver has been less extensively investigated than in muscle. Measurement of protein synthesis in the liver is complicated by the simultaneous synthesis of plasma proteins as well as its intracellular liver proteins. Moreover, the A–V balance approach is not possible, because of the difficulty of obtaining portal venous blood and, more importantly, because the liver is able to oxidize all circulating amino acids. Therefore, there is no amino acid that is a suitable representative of liver protein kinetics.

Early studies showed that in diabetes liver protein content was not affected (Kimball et al, 1989), whereas total serum protein concentrations were reduced, mainly due to a depression of albumin (McMillan, 1970; Jonsson and Wales, 1976). Recently De Feo et al (1991), using the precursor–product incorporation technique in insulin-deficient diabetic

humans, showed that albumin fractional synthetic rate was increased by insulin infusion while fibrinogen synthesis was reduced. With regard to intracellular liver proteins, using the constant-infusion technique, Pain and Garlic (1974) showed that fractional protein synthesis was unaffected in the liver of diabetic rats.

Regulation of protein breakdown in the liver has been investigated using the organ perfusion technique. The addition of either insulin (Mortimore and Mondon, 1970) or amino acids (Woodside and Mortimore, 1972) to perfusion of normal liver has an inhibitory effect on protein degradation. Interestingly, the insulin effect was not additive to the effect observed with a maximally effective level of amino acids (Neely et al, 1977; Mortimore et al, 1987). The role of amino acid availability in regulating liver protein breakdown independently from insulin was also suggested by studies showing that proteolysis in perfused liver from fed normal and diabetic mice was the same (Hutson et al, 1981). On the other hand, proteolysis was increased to a much greater extent in fasting diabetic animals than in controls.

It is well known that the gut, kidneys and skin play a major role in interorgan nitrogen exchange and whole body protein turnover. The fractional synthetic rates of intestinal mucosa, skin and kidney proteins are several times faster than those of muscle proteins (Waterlow et al, 1978). However, few data are available regarding the effect of insulin on tissues other than muscle and liver. In diabetic rats, gut and kidney protein content is significantly higher than those in controls (McNurlan and Garlic, 1981; Kimball et al, 1989). Therefore, further investigation is required to obtain a comprehensive description of the response of various tissues to insulin.

STATE OF INSULIN RESISTANCE

Many pathological conditions are characterized by resistance to the effects of insulin on glucose metabolism. Such metabolic abnormalities are most commonly present in type I and II diabetes, obesity, hepatic cirrhosis, Cushing's syndrome, trauma and sepsis. A number of studies have been performed to determine whether such resistance may also be extended to the insulin effects on protein and amino acid metabolism.

Diabetes

The hypothesis that resistance to the metabolic effects of insulin on protein metabolism might contribute to accelerated protein catabolism in poorly controlled type I diabetics has recently been tested. Type I diabetics need higher than normal insulin concentrations to maintain a normal or even higher rate of whole body proteolysis and leucine oxidation (Tessari et al, 1986a; Inchiostro et al, 1992). Moreover, the stepwise hyperinsulinaemic clamp decreases proteolysis to a lesser extent in diabetics than in normal subjects, either at physiological or pharmacological insulin concentrations (Tessari et al, 1986a). In other studies, the ability of hyperaminoacidaemia

with hyperinsulinaemia to stimulate protein synthesis in type I diabetics was also tested and found to be normal at the whole body level (Luzi et al, 1990; Inchiostro et al, 1992). In contrast, using either the precursor incorporation or the A–V balance technique, there was no effect on leg muscle protein synthesis in patients infused with insulin and amino acids (Bennet et al, 1990b, 1991). Taken together, these results demonstrate that type I diabetes is characterized by a resistance to the anabolic effects of insulin on protein metabolism, both at the whole body and the muscle level.

Very few studies of protein metabolism have been performed in patients with type II diabetes. Two studies have shown that in the postabsorptive state whole body protein turnover in type II diabetic patients is normal, whether they are treated with insulin (Staten et al, 1986) or diet only (Biolo et al, 1992). In addition, the ability of a mixed meal to reduce proteolysis is maintained in such patients despite the marked postprandial hyperglycaemia (Biolo et al, 1992).

Critical illness

Most aspects of the metabolic response are common to almost all critically ill patients. The catabolism of lean body mass is one prominent response (Wolfe et al, 1989). Also, glucose intolerance and the poor response of glucose metabolism to exogenous insulin administration are common (Wolfe et al, 1979; Shangraw et al, 1989). The concept of insulin resistance is therefore attractive in explaining the catabolic state in critically ill patients. However, in both patients with burns and those with sepsis who were resistant to the hypoglycaemic action of insulin, hyperinsulinaemia reduces whole body protein breakdown and leucine oxidation as effectively as in normal controls (Jahoor et al, 1989). These results indicate that insulin has differential sensitivity to glucose and protein metabolism in severe illnesses. However, further investigations are necessary to evaluate in stressed patients, the effect of insulin on muscle tissue, which is the main target organ of such catabolic response.

Hepatic cirrhosis and Cushing's syndrome

These diseases are also characterized by muscle wasting (Baxter and Forsham, 1972; McCullough and Tavill, 1991) and insulin resistance with regard to glucose metabolism (Rizza et al, 1982; Petrides and DeFronzo, 1989), as in sepsis and burns. However, the suppressive effect of insulin on whole body proteolysis is normal (Tessari et al, 1989; Petrides et al, 1991).

SUMMARY

On the basis of the preceding observations, the following sequence of events can be postulated during insulin deficiency or excess. The main feature of insulin deficiency is the disruption of protein balance in muscle that rapidly leads to emaciation and wasting. Muscle protein degradation is greatly

enhanced while increased amino acid availability maintains protein synthesis. In splanchnic tissues, both degradation and synthesis are increased but with an altered pattern, so that the levels of some proteins are increased (e.g. proteins of the acute-phase response), while those of others are decreased (e.g. albumin). As a result, intracellular protein content in liver is maintained but secretion of plasma proteins is abnormal. In healthy subjects, an acute increase in insulin concentration, as occurs after a meal, leads to a rapid suppression of protein breakdown in the splanchnic area. If hyperinsulinaemia is not supported by an exogenous amino acid supply, as might occur during a protein-free meal or experimentally during euglycaemic hyperinsulinaemic clamping, the plasma as well as muscle free amino acid concentration drops, owing to reduced splanchnic release. With reduced amino acid availability, insulin is not anabolic in muscle. If amino acid concentrations are maintained at normal or high levels, e.g. following a mixed meal, a net protein deposition in muscle may occur, primarily because of a stimulation of synthesis and possibly owing to inhibition of breakdown.

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